Faculty of Health & Life Sciences

Biomedical Sciences

Modulating Chemotherapeutic Drug Sensitivity and Counteracting Chemoresistance in Cancer using Frankincense

Molecular Biomedicine

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy at De Montfort University

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Abstract

The development of resistance to chemotherapy is a recurrent and serious problem applicable to many types of cancer including ovarian cancer, which can show intrinsic and acquired resistance to first-line chemotherapy such as platinum-based drugs (e.g. cisplatin, carboplatin) and taxanes (e.g. paclitaxel), and for the anthracyclines (e.g. doxorubicin).

Plants have been a source of therapeutic agents for various diseases for thousands of years, the taxanes being an example. Frankincense (from *Boswellia sp.*) has been used in traditional medicine for centuries. Bioactive components (primarily boswellic acids) responsible for the therapeutic actions of frankincense have been investigated and characterised in many cases, although activity against selected cancer types, ability to synergise with existing therapies and to overcome chemoresistance is not as well explored. The identification of individual components in frankincense that can synergise or otherwise yield unexpected anti-cancer effects may yield useful information that can be exploited for therapy and give additional insight into mechanisms of cancer chemoresistance.

This project explores the potential utility and mechanisms of action of 3-O-acetyl-11-keto-β-boswellic acid (AKBA), a bioactive component of frankincense, in counteracting chemoresistance in cancer, using ovarian cancer as the primary model. The work was conducted *in vitro* using sensitive and resistant ovarian cancer cell lines and a range of cell and molecular biology approaches to determine the effect and mechanisms of action of AKBA on ovarian cancer cells. This included cell viability assay, cell cycle analysis, mitochondrial membrane potential ($\Delta\psi_m$) assay (apoptosis) and protein expression analysis to help examine mechanism of action of AKBA.

The key finding of this study is the synergistic interaction between AKBA and doxorubicin, which could consequently lead to reduction of doxorubicin dose and the side effects,. The difference between the EC_{50} values of doxorubicin in the presence or absence of AKBA were statistically significant. Alterations in the $\Delta\psi_m$ and the inhibition of NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) pathway by supressing the expression of multiple proteins seem to be among the mechanisms of action behind the ability of AKBA to sensitise cancer cells to doxorubicin. An apparent antagonistic interaction was reported between AKBA and cisplatin, on A2780, A2780cis and OVCAR4 cells. The same was observed with paclitaxel on A2780cis, however, the resulting interaction on A2780 was additive. Further studies to understand or overcome these antagonistic interactions are

required. Despite the limitations, current findings indicate that bioactive components of frankincense could help in overcoming resistance in cancer cells and enhance the efficacy of selected chemotherapy drugs.

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Poster presentation

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List of abbreviations

- 5-LO 5-lipoxygenase
- A2780 Ovarian endometroid adenocarcinoma cells
- A2780ADR Doxorubicin resistant A2780
- A2780cis Cisplatin resistant A2780
- ABA 3-O-acetyl-β-boswellic acid
- ABCF2 ATP-binding cassette subfamily F member 2
- AEG-1 Astrocyte elevated gene 1/metadherin
- AKBA 3-O-acetyl-11-keto-β-boswellic acid
- Akt Protein kinase B (PKB)
- APAF1 Apoptotic protease -activating factor-1
- ARE Antioxidant-response element
- ARID1A AT-rich interactive domain-containing protein 1A
- ASC Apoptosis-associated speck-like protein containing a CARD
- ATP Adenosine triphosphate
- BA Boswellic acid
- BCL-10 B-cell lymphoma/leukemia 10
- BCRP/ABCG2 Breast cancer resistance protein
- BRAF B-Raf proto-oncogene serine/threonine-protein kinase
- BRCA1/2 Brest cancer genes 1 and 2
- BSE Boswellia serrata extract
- CARD6 Caspase recruitment domain-containing protein 6
- CCC Clear-cell carcinoma
- CCRF-CEM lymphoblastic leukemia
- CD40/TNFRSF5 Cluster of differentiation 40/TNF receptor superfamily member 5
- Cdc Cell division control protein
- CDK Cyclin-dependent kinase
- CDK12 Cyclin-dependent kinase 12
- CI Combination index
- cIAP1/2 Cellular inhibitor of apoptosis protein 1/2
- CML chronic myeloid leukemia

- COX-2 cyclooxygenase 2
- CSC Cancer stem cells
- CTNNB1 Catenin (cadherin-associated protein), beta 1
- CXCR4 C-X-C Motif Chemokine Receptor 4
- CYPs Cytochrome P450 enzymes
- DCFDA 2',7' –dichlorofluorescin diacetate
- DMSO Dimethyl sulfoxide
- DNA Deoxyribonucleic acid
- DR5 Death receptor 5
- EC Endometrioid carcinoma
- EOC Epithelial ovarian carcinoma
- ER/UPR Endoplasmic reticulum/unfolded protein response
- FADD FAS-associated death domain protein
- FasR/Fas Fas cell surface death receptor
- FDA Food and Drug Administration
- FEc/FEo Clear frankincense extract/oily frankincense extract
- FTH1 Ferritin heavy chain
- FTL Ferritin light chain
- FUCCI fluorescence ubiquitination cell cycle indicator
- G6PD Glucose-6-phosphate dehydrogenase
- GCL Glutamate cysteine ligase
- GPx Glutathione peroxidase
- GSH Glutathione
- GSTA2 Glutathione S-transferase A2
- GSTs Glutathione S-transferases
- H_2O_2 Hydrogen peroxide
- HCT-116 Human colon carcinoma cells
- Hep G2 Human hepatocellular carcinoma cells
- Hep3B Human hepatocellular carcinoma cells
- HER2 Human epidermal growth factor receptor 2
- HGSOC High-grade serous ovarian cancer
- HL-60 Human leukaemia cells
- HNF1 Hepatic nuclear factor 1

- HNPCC	Hereditary nonpolyposis colorectal carcinoma
- HO-1	Heme Oxygenase 1
- HPLC	High performance liquid chromatography
- HRP	Horseradish peroxidase
- HT29	Human colorectal adenocarcinoma cell line
- IKK	I kappa B kinase
- IKK	Inhibitor of nuclear factor kappa-B kinase
- IL-1 R1	Interleukin 1 receptor1
- IL-17 RA	Interleukin 17 receptor A
- IL-18 R alpha	Interleukin 18 receptor alpha
- IL-6	Interleukin 6
- IRAK1	Interleukin 1 receptor associated kinase 1
- IRF5	Interferon regulatory factor 5
- IRF8	Interferon regulatory factor 8
- IV	Intravenous
- IκB inhibitor	Nuclear factor of kappa light polypeptide gene enhancer in B-cells
- JNK	c-Jun N-terminal kinase
- KBA	11-keto-β-boswellic acid
- KRAS	Kirsten rat sarcoma viral oncogene homolog
- LGSOC	Low-grade serous ovarian carcinoma
- LLOQ	Lower limit of quantitation
- LNCaP	Human prostate adenocarcinoma cells
- LoVo	Human colorectal adenocarcinoma cells
- LS174T	Human colorectal adenocarcinoma cell line
- LTBR/TNFRSF3 superfamily member 3	Lymphotoxin-beta receptor/tumour necrosis factor receptor
- M1	A subtype of macrophages
- MC	Mucinous carcinoma
- MDA-MB-231	Adenocarcinoma cells
- MDR1/ABCB1/P-gp	Multi drug resistance gene/ Permeability glycoprotein

- MMP-9 Matrix metallopeptidase 9
- MRPs Multidrug resistance associated proteins
- MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
- MYD88 Myeloid differentiation primary response 88
- NCS Newborn calf serum
- NF-κB Nuclear factor kappa-light-chain enhancer of activated B-cells
- NF1 Neurofibromin 1
- NFκB Nuclear factor kappa-light-chain-enhancer of activated B cells
- NGF R Nerve growth factor receptor
- NQO1 NAD(P)H: quinine oxidoreductase 1
- NQO1 NADPH: quinone oxidoreductase 1
- Nrf2 Nuclear factor e2-related factor 2
- OVCAR4 Ovarian adenocarcinoma cell line
- p53 Tumour protein p53
- PBS Phosphate buffered saline
- PC-3 Human prostate cancer cells
- PC3/Doc Human prostate cancer cells (docetaxel-resistant)
- PI Propidium iodide
- POI3KCA Phosphatidylinositol 3-kinase
- PPP2R1A Protein Phosphatase 2 (Formerly 2A), Regulatory Subunit A (PR 65), Alpha Isoform
- Prx1 Peroxiredoxin 1
- PTEN Phosphatase and tensin homolog
- Rb Retinoblastoma protein
- RF Resistance factor
- RNA Ribonucleic acid
- ROS Reactive oxygen species
- SEM Standard error of the mean
- SHARPIN Shank-associated RH domain-interacting protein
- SK-OV-3 Ovarian adenocarcinoma
- SOCS-6 Supressor of cytokine signalling 6
- Srx1 Sulfiredoxin 1
- Stat Signal transducer and activator of transcription

- STING Stimulator of interferons genes
- TBS Tris buffered saline
- TLC Thin layer chromatography
- TNF Tumour necrosis factor
- TRAIL TNF-related apoptosis-inducing ligand
- TRAIL R2 Tumour necrosis factor-related apoptosis-inducing ligand receptor 1
- Trx1 Thioredoxin 1
- TrxR Thioredoxin reductase
- Trypsin-EDTA Trypsin-ethylenediaminetetraacetic Acid
- UGT Uridine 5′-diphosphoglucuronosyltransferase
- UV Ultraviolet
- UWB1.289 BRCA1-null human ovarian cancer cell line
- VEGF Vascular endothelial growth factor
- WHO World Health Organisation
- β-BA β-boswellic acid
- $\Delta\psi_m$ or MMP Mitochondrial membrane potential

Chapter 1

Introduction

1 Introduction

1.1 Cancer

In 2012 and 2013 the incidence rate of cancer was 14 million worldwide (WHO, 2017) and more than 300,000 new cases in the UK (Cancer Research UK, 2013). More than 8 million deaths were reported to be caused by cancer around the world in 2012, where lung cancer was ranked at the top of the list as a leading cause of about 1.5 million deaths (WHO, 2017). Understanding cancer is crucial for prevention and to improve quality of life and survival rates of patients.

Cancer is a complex genetic disease initially and simply defined as uncontrolled cellular growth. Cancer develops as a complex multi-step process, illustrated and expanded by Hanahan and Weinberg (2000, 2011), who introduced the hallmarks of cancer that can be divided into two categories: enabling and ability characteristics (Petrillo *et al.*, 2016) (see Table 1.1). In addition, the complexity of cancer increases by knowing that more than one cell type is involved in the growth and maintenance of a tumour and contributing to tumour progression forming what is widely known as the "tumour microenvironment" (see Figure 4 in Hanahan and Weinberg (2011)). This complexity could be one of the reasons behind chemoresistance and cancer recurrence.

Table 1.1: Categorisation of the hallmarks of cancer

Cancer enabling characteristics	**Cancer ability characteristics**
Genomic instability	Sustained proliferative signaling
Inflammatory state	Evasion of growth suppressors
	Evasion of immune destruction
	Replicative immortality
	Resistance to cell death
	Angiogenesis
	Activation of invasion and metastasis
	Energy metabolism

* Based on Petrillo *et al.* (2016) and Hanahan and Weinberg (2000, 2011)

1.1.1 Ovarian cancer

Understanding of epithelial ovarian cancer (EOC) is crucial considering its high mortality rate. It is the 4th leading cause of cancer related deaths in women in developed countries (Jayson *et al.*, 2014) and the primary cause of gynaecologic cancer related deaths in women (Bagnato and Rosanò, 2012). In 2014, more than 7,000 women were diagnosed with ovarian cancer in the UK, where the reported number of deaths was more than 4,000 cases (55 %) (Cancer Research UK, 2014). The estimated figures for ovarian cancer in 2017 in the USA are 22,440 new cases with 14,080 deaths caused by the disease (American Cancer Society, 2017). Ovarian cancer can originate from the epithelium (~ 90%), germ cells (~ 5%) and stromal cells (~5%) (Figure 1.1), however, the topic is still debatable (Shield *et al.*, 2009). The following sub-types of EOC are recognised: serous, endometrioid clear, cell mucinous, or undifferentiated/unclassifiable (Cancer Research UK, 2016). The current histologic classification of EOC (Table 1.2) for research purposes are type I and type II. Type I tumours include low-grade serous, low-grade endometrioid cancers, mucinous, clear cell, and transitional cell carcinomas. Type II include high-grade serous, high-grade endometrioid carcinomas, carcinosarcomas and undifferentiated carcinomas, which are known for their aggressiveness with late stage diagnosis (Cobb *et al.*, 2015). It is important to indicate that peritoneal cancer and fallopian tube cancer are considered clinically as ovarian cancers for the shared similarities, which are also involved in the ovarian cancer staging (Prat, 2014).

Table 1.2: Characteristics of common EOC

	HGSOC	LGSOC	MC	EC	CCC
Incidence % of EOC	70 %	<5 %	3 %	10 %	10 %
Risk Factors	BRCA1/2	-	-	*HNPCC	-
Precursor lesions	Tubal intraepithelial carcinoma	Serous borderline tumour	Cystadenoma/borderline tumour	Atypical endometriosis	Atypical endometriosis
Pattern of spread	Very early peritoneal dissemination	Peritoneal dissemination	Usually confined to ovary	Usually confined to pelvis	Usually confined to pelvis
Associated mutations	BRCA, p53, [a]NF1 [a]CDK12	BRAF, KRAS	KRAS, HER2	PTEN, ARID1A, [a]PI3KCA, [a]PPP2R1A	HNF1, ARID1A, [a]PI3KCA, [a]PTEN, [a]CTNNB1, [a]PPP2R1A

HGSOC, high-grade serous ovarian carcinoma; LGSOC, low-grade serous ovarian carcinoma; MC, mucinous carcinoma; EC, endometrioid carcinoma; CCC, clear-cell carcinoma.
*Hereditary nonpolyposis colorectal carcinoma.
[a](Jayson *et al.*, 2014).
Adapted from Prat (2012).

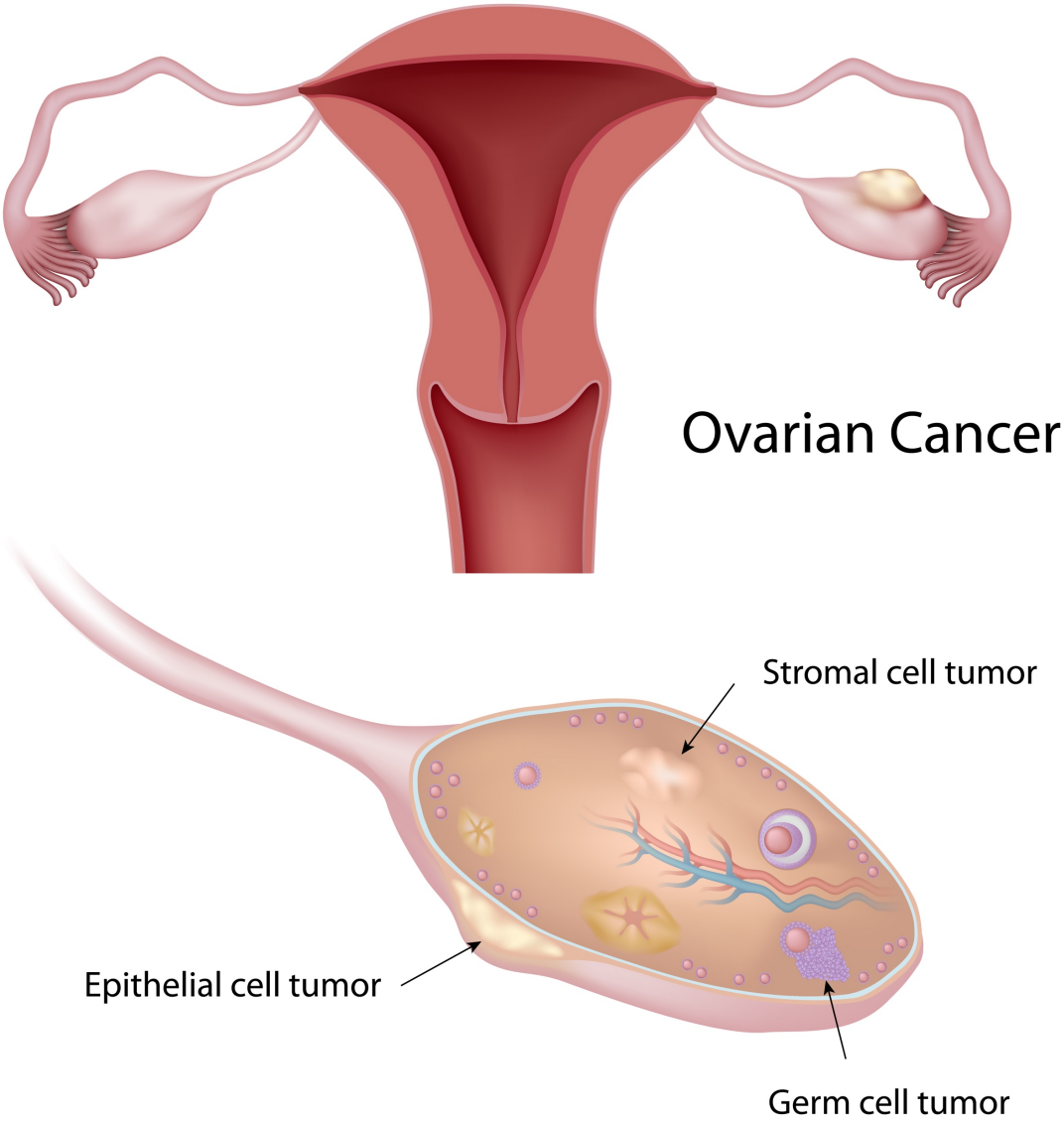


Figure 1.1: Ovarian cancer. *(Shutterstock ID: 147789488)*

Where EOC is diagnosed late in an advanced stage (stage III), EOC is difficult to treat (Jayson *et al.*, 2014). Although the detection of the disease in earlier stages has shown certain improvements in the last few years, EOC disseminates in the early stages to pelvic and abdominal organs and in an unusual way compared to the other epithelial cancers, in that EOC does not usually require the vasculature to disseminate (Shield *et al.*, 2009). In addition, the hallmarks of cancer have been successfully applied for ovarian cancer. Ovarian cancer is a heterogeneous group of related diseases with distinctive molecular profiling and histological variations (Goff, 2013). This information gives an idea of the prospects for survival time that may result from EOC and how urgent it is to develop protective strategies

and diagnostic techniques, which could reduce the number of patients and help to improve the survival rate.

1.1.2 Ovarian cancer treatment

Similar to other types of cancer, radiotherapy, surgery and chemotherapy are the key strategies to treat ovarian cancer. First line chemotherapy is usually given every two to three weeks as a combination of a platinum compound, such as cisplatin, and a taxane, such as paclitaxel (Bell and Lutman, 2010). The development of resistance to chemotherapy is a recurrent and serious problem applicable to many types of cancer; ovarian cancer is a particular tumour type that can show intrinsic and acquired resistance to first-line chemotherapy (Kigawa, 2013). Recurrent ovarian cancer is usually sensitive to platinum drugs. Therefore, a combination of platinum containing drugs and liposomal doxorubicin for example is recommended for the recurrent disease (Jayson *et al.*, 2014). Armstrong *et al.* (2012) stated that it is more difficult to overcome chemoresistance that arises from combined platinum agent and taxane therapy.

The new advances in the understanding of the distinct molecular characteristics of EOC histologic sub-types is leading to promising molecularly-targeted treatments. This may lead to more selective and less toxic agents compared to current chemotherapies (Banerjee and Kaye, 2013). As stated by Banerjee and Kaye (2013), the historic approaches of treatment considered the sub-types as one entity despite the molecular differences which emerged later.

1.1.3 The molecular mechanisms of action of cisplatin, paclitaxel and doxorubicin

Exploring the mechanisms of action of chemotherapies could help in understanding and overcoming chemoresistance in tumours. The mechanisms of action are usually complex and, in some cases, unclear and need further studies to provide the closest possible explanation of the agents interaction in the body. This is probably a result of the involvement of a number of molecular and cellular changes and even biologically active metabolites in the action of chemotherapies. A brief exploration of the mechanisms of action of cisplatin, paclitaxel and doxorubicin will be mentioned below.

1.1.3.1 Cisplatin

Cisplatin (cis-Diamminedichloroplatinum(II)) (Figure 1.2) is a well known potent anti-tumour agent that was accidently discovered from platinum electrodes in the 1960s. Dr. Rosenberg and his colleagues noticed that E. coli bacteria stopped dividing, when they run electrical current in a solution that contains the bacteria using platinum electrodes. It was first used as an antibacterial agents then was shown to be effective against tumours. The first reported clinical trial was conducted in 1972 where a number of undesirable serious toxic effects to the kidney and the bone marrow, for example, were observed. This has led to further investigations and developments to the agent and the treatment regimens which resulted in the generation of one of the leading anti-cancer agents that was registered in USA in 1978, and in Europe and Japan in 1984 (Reedijk and Lohman, 1985; Makovec, 2019). Cisplatin has been particularly used to treat bladder, head, neck, lung, ovarian, and testicular cancers. It is also potent against various types of cancers including carcinomas of various tissue/cell type origin, germ cell tumours, lymphomas, and sarcomas.

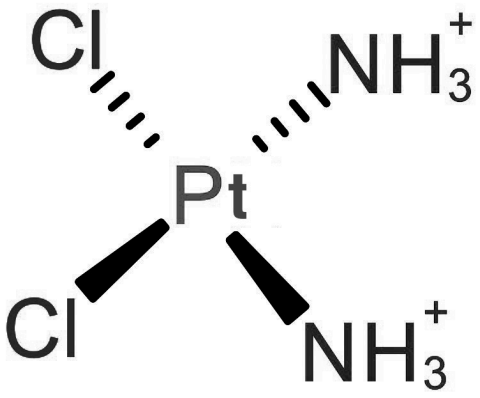


Figure 1.2: Cisplatin chemical structure.

A number of molecular mechanisms, protein and genetic alteration play a role in the toxicity caused by cisplatin and its metabolites towards cancer cells. Cisplatin leads to DNA damage by various methods including crosslink with the purine bases on the DNA and interferes with DNA repair mechanisms which causes DNA damage, blocks DNA replication and induces apoptosis in cancer cells. Increasing cellular oxidative stress is another mechanism of cisplatin toxicity which target the mitochondria and leads to loss of mitochondrial protein sulfhydryl groups, calcium uptake inhibition and reduction of mitochondrial membrane potential (Shaloam and Tchounwou, 2014). Cisplatin also affects multiple protein signalling pathways.

1.1.3.2 Doxorubicin

Doxorubicin (adriamycin) (Figure 1.3) is another good example of the medicines that were extracted from natural sources. It belongs to a family of antibiotics and chemotherapies called anthracycline. The discovery of doxorubicin goes back to the late 1960s from a mutant strain of *Streptomyces peucetius*. Discovery occurred when researchers worked on developing new strains of *S. peucetius* looking for new antitumor agents following the success of the isolation of daunorubicin from the wild *S. peucetius* that showed clinical importance for leukaemia (Arcamone *et al.*, 1969; Lomovskaya *et al.*, 1999).

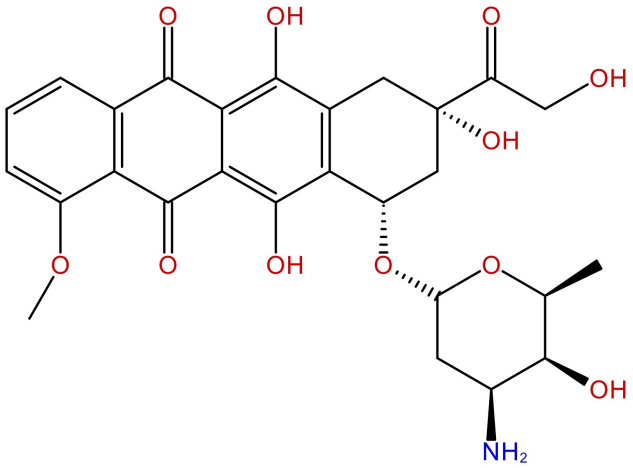


Figure 1.3: Doxorubicin chemical structure

Similar to other chemotherapies, the mechanism of action of doxorubicin is complex and affects a number of molecular targets. The commonly known mechanisms involve the interfering with DNA synthesis through intercalation and inhibiting topoisomerase II. Stabilising topoisomerase II prevents resealing the double helix DNA leading to the interruption of the DNA replication. The other mechanism is the induction of free radicals that causes DNA and cellular damage, where reduction or oxidation of doxorubicin can induce intracellular hydrogen peroxide generation (H_2O_2). These mechanisms in addition to membrane lipid oxidation and the inhibition of the respiratory chain enzymes in mitochondria lead to the induction of apoptosis (Müller *et al.*, 1997; Mizutani *et al.*, 2005; Pilco-Ferreto and Calaf, 2016; Falzone, Salomone and Libra, 2018).

1.1.3.3 Paclitaxel (Taxol®)

The history of discovering paclitaxel (Figure 1.4), among other plant based medicines, serves as an example that indicates to the importance of plants as a source of therapeutic agents. Paclitaxel was extracted from the bark of *Taxus brevifolia* (Pacific Yew) in the USA as part of a screening program that was exploring the plant kingdom for antitumor agents which started in 1960. Drs Wall and Wani were interested in studying the bark of *T. brevifolia* following their earlier success in isolation of camptothecin from a Chinese tree (Happy Tree), *Camptotheca acuminata*, which was potent against L1210 mouse leukaemia cell line *in vitro*. Taxol is a secondary metabolite that is isolated through bioactivity-guided determination in a multi-step isolation process. After a decade of discovering and developing the agent, Taxol® was used to treat ovarian cancer which was then followed by reporting its efficacy against breast, lung and prostate cancers (Wall and Wani, 1996).

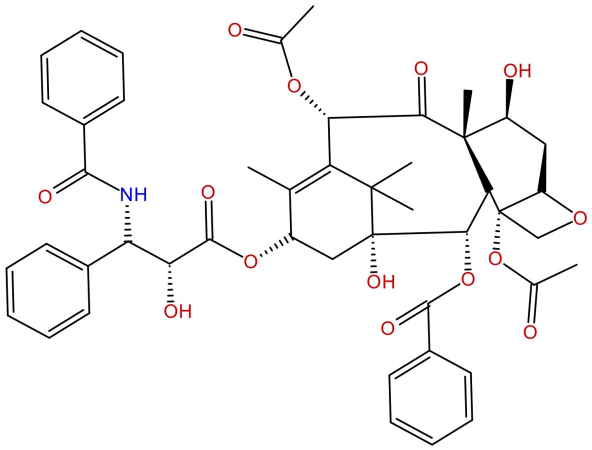


Figure 1.4: Paclitaxel chemical structure

When it comes to the mechanism of action, paclitaxel arrests cell cycle at G2/M phase by stabilizing microtubules and inhibiting their depolymerisation to tubulin which in turns blocks mitosis (Wall and Wani, 1996) through activating the mitotic checkpoint. This observation was reported by a number of researchers at high doses of paclitaxel in cell culture, nonetheless the apparent mechanism was the induction of multipolar spindles in breast cancer cell lines when clinically relevant doses were tested, 5-50 nM in cell lines to 1-9 µM intratumoral injection in patients (Weaver, 2014).

1.1.4 Chemoresistance

Chemotherapy, in addition to the other two strategies: surgery and radiotherapy, is a major component of the conventional treatment of cancer. However, the efficacy of chemotherapy is limited due to the ability of cancer to resist the therapy, which can be intrinsic or acquired (Wilson, Longley and Johnston, 2006; Chang, 2011). While the acquired resistance occurs due to the genetic adaptation of cancer cells in response to the stress that imposed by chemotherapies, intrinsic resistance can arise before or during the early stages of tumorigenesis without the need for cells to be completely transformed. Expression of hTERT (Telomerase reverse transcriptase) and inactivation of pathways that are regulated by pRb and p53 are the minimum genetic alterations that allow primary cells to be chemoresistant (Raguz and Yagüe, 2008). Acquired resistance in solid tumours could potentially occur within a short period of time (hours/days) following the treatment, which involves overexpression of resistance genes such as multi-drug resistance gene I (MDR-I), also known as permeability glycoprotein (P-gp), or down-regulation in target genes, e.g. topoisomerase IIα (Di Nicolantonio *et al.*, 2005). P-gp expression and function was increased after 16 hours of *ex vivo* exposure of acute and chronic myeloid leukemic blasts to a number of anthracycline analogues or cytosine arabinoside (Hu *et al.*, 1999). The level of MDR-I RNA was increased following 50 minutes *in vivo* exposure to doxorubicin in patients with metastatic sarcoma (Abolhoda *et al.*, 1999). P-gp role in chemoresistance will be discussed in chapter 5.

Chemoresistance contributes to the treatment failure in more than 90 % of the cases of metastasised disease (Wilson, Longley and Johnston, 2006), which indicates to the importance of understanding of the mechanisms behind this phenomenon. Efflux of the chemotherapeutic agent from the tumour cell; modification of drug targets; changes or mutation in mitotic checkpoint signals; drug sequestration; de-toxification of cytotoxic agents; and enhanced DNA repair mechanisms are the cellular mechanisms that have been established in chemoresistance of cancer to a number of therapeutic agents (Chang, 2011). One or more of these mechanisms could be found in a particular tumour type because of cellular heterogeneity in cancer (Vasey, 2003).

Most of the available cytotoxic chemotherapies target cells that are rapidly dividing, which could be one of the reasons that leads to cancer relapse and chemoresistance. Changes in the rate of proliferation were investigated and reported to be lower, in many cases of single and dual resistant cancer cell lines, than their sensitive counterpart (Armstrong *et al.*, 2012). In

addition, it was demonstrated that invading cells are in phases G0/G1 of mitosis using real-time FUCCI (fluorescence ubiquitination cell cycle indicator) imaging (Yano *et al.*, 2014).

On the other hand, cytotoxic drugs are therapeutically effective against solid tumours, even though it was reported that solid tumours are generally slow in terms of proliferation. This paradox was explored by Mitchison (2012) using paclitaxel on solid tumours in mice. It was reported that cells died after days of treatment despite the fact that they had not entered mitosis nor were they proliferating, whereas the expected result by induction of mitotic arrest followed by apoptosis was observed in less than 25 % of cells. This brings forward the recent concept of quiescent, so called cancer stem cells (CSC), cells that was introduced as one of the reasons for chemoresistance.

1.1.5 Cancer stem cells (CSCs)

Recently, the hypothesis of the existence of CSCs in cancer and their role in cancer initiation and the ability of cancer to resist chemotherapies has been under investigation. In the original model for tumour initiation, every single cell in a heterogeneous tumour has the ability to regenerate a tumour which is called the stochastic model. The other model is called hierarchical or CSC model, where the ability to initiate a tumour is possessed by a small population of cancer cells (Tirino *et al.*, 2013). This population could be a result of genetic and/or epigenetic alterations of adult stem cells, which enable them to transform and metastasise to other organs or tissues (Davis, Tinker and Friedlander, 2014).

The presence of CSCs in various types of cancers such as chronic myeloid leukaemia (CML), brain and gastrointestinal tumours was demonstrated in several studies (Raguz and Yagüe, 2008). Stem cells were also found in ovarian cancer and were related to its aggressiveness (Bapat *et al.*, 2005). To differentiate CSCs from other cells in cancer, a number of defining characteristics were stated. This includes enhanced tumour initiating potential and stem-like properties that are self-renewal and the ability to differentiate to another tumour cell sub-type (Abdullah and Chow, 2013). In addition, the ability of CSCs to remain quiescent for a long time, and survive the harsh environments and cytotoxic drug insult indicate to the possible role of CSCs in chemoresistance and tumour relapse (Tirino *et al.*, 2013). Enhanced DNA repair mechanisms and expression of detoxifying aldehyde dehydrogenase enzymes are among the mechanisms that were reported for CSCs to resist chemotherapies (Thomas *et al.*, 2014).

1.1.6 The side effects of chemotherapies

Despite the fact that chemotherapies have been and still are one of therapeutic approaches to treat cancer, there are a number of serious undesirable side effects on normal cells and tissues. Numerous side effects are linked to cisplatin including, but not limited to, neurotoxicity and nephrotoxicity. Nephrotoxicity is noticeable and clinically significant where developing acute renal failure is estimated to happen in about 30 % of the patients after receiving dose of 50 -100 mg per/m^2 cisplatin (Barabas *et al.*, 2008; Barton *et al.*, 2018). With regards to paclitaxel, the main side effects include serious hypersensitivity, nephrotoxicity and neurotoxicity (Koudelka and Turánek, 2012). Finally, the major known side effect of doxorubicin is cardiotoxicity (Wortman *et al.*, 1979; Kalyanaraman *et al.*, 2002; Hershman *et al.*, 2007). Therefore, a number of approaches have been established and are still under development and investigation to reduce the side effects of chemotherapies. This includes, for example, tumour targeted therapies, natural and synthetic adjuvants and nanoparticles.

1.2 Frankincense

Plants have been a source of therapeutic agents for various diseases for thousands of years, the taxanes, leading to the development of tamoxifen for the treatment of breast cancer being an example. Traditional medicine is based predominantly around the use of selected plant components delivered as mixtures, often taken orally. Frankincense (from *Boswellia sp.*) (Figure 1.5) has been used in traditional medicine for centuries. It has been used to treat 'fever, skin diseases, rheumatism, respiratory diseases including laryngitis and cough with copious amounts of sputum, liver disorders, ringworm, boils, strengthening of teeth, healing eye inflammations, enhancing the growth of hair, improving appetite, wound healing, soothing mouth sores, improving diarrhoea, general restorative, tonic effects and more' (Rentea, 2008). Frankincense is a French word that means pure incense which has also been used in religious and cultural events in various cultures and societies (Ammon, 2006). Indian olibanum, Salai guggal, Loban, or Kundur are other popular names of frankincense. The trunk of Boswellia trees produces an oleogum resin in response to scrapes or wounds to prevent infection and heal (Garg and Deep, 2016).

Figure 1.5: Boswellia tree and frankincense olibanum resin. *(https://commons.wikimedia.org)*

1.2.1 Composition and biological activity

Oleogum resin contains more than 200 compounds with variability in terms of contents depending on the species, harvestings and the tree location (Ammon, 2006). Volatile oils (5–15%), pure resin (55–66%) and mucus (12–23%) are the main components of oleogum resin (Tawab *et al.*, 2011). Triterpenes, such as pentacyclic and tetracyclic triterpenes, are among the terpenes that compose the resin. Pentacyclic bioactive components (primarily boswellic acids, BAs) responsible for the therapeutic actions of frankincense have been investigated and characterised in many cases (Hamidpour et al., 2016), although activity against selected cancer types, ability to synergise with existing therapies and to overcome chemoresistance is not as well explored.

β-boswellic acid (β-BA), acetyl-β-boswellic acid (ABA), 11-keto-β-boswellic (KBA) acid and 3-O-acetyl-11-keto-β- boswellic acid (AKBA) are examples of the main bioactive boswellic acids (BAs) that have been found in frankincense (Table 1.4). Inhibition of pro-inflammatory enzymes has been reported to be one of the effects of these boswellic acids (Hamidpour et al, 2016). AKBA, in particular, has shown a strong anti-inflammatory effect by inhibiting 5-lipoxigenase, which has been found to be overexpressed in a number of chronic diseases including chronic inflammation, cardiovascular diseases and cancers (Rentea, 2008). Moreover, inhibitors of 5-LO are suggested to be part of chemoprevention strategies of various types of cancers due to its contribution to cell proliferation and apoptosis inhibition (Garg and Deep, 2016). In contrast to AKBA and KBA, which are selective, potent, non-redox and non-competitive inhibitors of 5-LO, most available enzyme inhibitors lead to undesirable side effects for being non-selective (Kaunzinger *et al.*, 2002). Despite this selectivity and potentiality towards 5-LO *in vitro*, the pharmacological relevance of this effect *in vivo* is implausible (Siemoneit *et al.*, 2009), which could be attributed to the issues related to the bioavailability of AKBA (see 1.2.3).

In addition to BAs, essential oils have been reported to be cytotoxic towards tumour cells. For example, Ni *et al.* (2012) demonstrated the cytotoxicity of various fractions of essential oils, that have been prepared from hydrodistillation of *Boswellia sacra* gum resins, on pancreatic cancer. Essential oils in the fractions with high molecular weight were toxic to and induced apoptosis through caspase dependent pathway on pancreatic cancer cells. Although the fractions with high concentration of BAs (19.6 and 30.1 mg/mL) were considered more potent than the others (0.81 and 0.91 mg/mL), the latter were reported to be cytotoxic. This could mean that there are anti-tumour compounds in frankincense other than BAs.

1.2.2 The molecular targets of boswellic acids

There are multiple established molecular targets of boswellic acids in chronic diseases, including cancer, which could be linked to their various biological activities. The targets are enzymes, growth factors, transcription factors, kinases and others involved in cell survival and proliferation (Table 1.3). For example, AKBA arrested cells at G1 and downregulated cyclin-dependent kinases (CDKs) through p53-independent upregulation of p21 CDK inhibitor in colon cancer cell lines, HCT-116, HT29 and LS174T (Khan *et al.*, 2016; Roy *et al.*, 2016, 2019). This variation and multiplicity could explain the potentiality of boswellic acids and frankincense extracts in cancer prevention and treatment. The multiplicity of molecular mechanisms targeted by boswellic acids on a variety of cancer cell lines was reported on a genetic level using mRNA-based microarray, COMPARE (Eichhorn, Greten and Efferth, 2011).

Table 1.3: Non exhaustive list of published molecular targets of Boswellia serrata extract (BSE) and BAs in cancer

Extracts/Compound	**Cancer type and used model**	**Molecular target/Mechanism**	**Reference**
BSE and/or ABA	Breast cancer: MDA-MB-231 cells	↑ ER/UPR response	(Mazzio, Lewis and Soliman, 2017)
KBA and AKBA	Liver cancer: Hep G2 cells	↑ Caspase-3 & -8	(Liu *et al.*, 2002)
BSE	Liver cancer: HepG2 and Hep3B cells	↑ caspase-3, TNF-α and IL-6	(Khan *et al.*, 2014)
AKBA	Pancreatic cancer: several cell lines and a mouse model	↓ NFκB , NFκB regulating genes, COX-2, MMP-9, CXCR4, and VEGF	(Park *et al.*, 2011)
BSE and AKBA	Colorectal carcinoma: LoVo and HT29 cells	↓ NFκB phosphorylation	(Catanzaro *et al.*, 2015)
AKBA	Prostate cancer: PC-3 and LNCaP cells	↑ Caspase-3 & -8, DR5 and PARP cleavage	(Lu *et al.*, 2008)
AKBA	Prostate cancer: PC-3 cells	↓ IKK and NFκB	(Syrovets *et al.*, 2005)
AKBA	Prostate cancer: PC3/Doc (docetaxel-resistant)	↓Akt and Stat 3	(Liu *et al.*, 2019)
AKBA	Leukemia: HL-60 and CCRF-CEM cells	↓ Topoisomerases I	(Hoernlein *et al.*, 1999)

1.2.3 Bioavailability of natural products

The poor bioavailability of natural bioactive compounds and herbal extracts following oral administration is one of the main challenges that limit the use of natural remedies in clinical settings. Oral administration is widely preferred over other routes, such as intravenous (IV), for the following advantages: painlessness, easy self-administration, high patient compliance, and feasibility for outpatients (Lin *et al.*, 2017). Solubility and/or dissolution, permeation, first-pass metabolism, and pre-systemic excretion or efflux are the oral bioavailability barriers (Hu and Li, 2011). Despite the promising reports of a number of a number of natural products as cancer chemopreventive agents discussed in the review by Gao *et al.* (2013), the bioavailability of the agents *in vivo* and in pre-clinical studies was much lower than the

required doses to exert the desired effects (Gao *et al.*, 2013). This was linked to one or more factors such as poor solubility in water and/or rapid/extensive metabolism.

A number of approaches were investigated to enhance the oral bioavailability of natural products based on the understanding of the reasons behind the poor oral bioavailability of a particular agent. This include, non-exhaustively, adjuvants, nanoparticles, and liposomes, micelles, and phospholipid complexes, and analogues and derivatives (Anand *et al.*, 2007; Gupta *et al.*, 2013). For example, curcumin, a bioactive component from turmeric, bioavailability was reported to increase by 2000 % when it was combined with piperine, a bioactive component from black pepper (Gupta *et al.*, 2013; Hewlings and Kalman, 2017). Further research is still needed to overcome this issue.

The discussed poor oral bioavailability of natural products propose the question about the efficacy of using other administration routes, such as IV, intraperitoneal and topical application…etc., on natural compounds bioavailability. Poor solubility, rapid metabolism and/or elimination, as examples, stand as obstacles against achieving required doses despite the reported improved plasma concentration and area under curve (AUC) of some natural compounds after IV administration compared to oral administration (Das *et al.*, 2008; Kapetanovic *et al.*, 2011; Estrela *et al.*, 2017). Taking into consideration the aforementioned challenges of IV administration of natural products, it could be used to avoid gastrointestinal barriers, although primary tumours of the gastrointestinal tract could be treated with effective natural products orally (Estrela *et al.*, 2017). In addition, it was reported that topical application of resveratrol, a naturally occurring polyphenol, could protect against ultraviolet radiation-induced skin cancer (Xiao *et al.*, 2019).

The solubility of natural bioactive compounds is one of the challenges that limits their bioavailability. Dimethyl sulfoxide (DMSO) is a solvent that is wieldy used to dissolve a broad range of both polar and non-polar poorly soluble compounds. It is also commonly used as a vehicle and/or a therapeutic agent in laboratory studies and clinical practice. This includes, non-exhaustively, cryopreservation, free radical scavenging, inducing anti-inflammation, muscle relaxation and penetration enhancer in topical application. DMSO is generally considered nontoxic if it is below 10 % (v/v) and is classified as class 3 with ethanol and other solvents by United States Food and Drug Administration (FDA) in spite of the lack of full understanding of its physiological and pharmacological effects (Santos *et al.*, 2003; FDA, 2012; Costa *et al.*, 2017; Verheijen *et al.*, 2019).

On the other hand, a number of side effects and drug interactions were reported questioning the safety of DMSO. The reported systemic side effects of DMSO includes nausea, vomiting, diarrhea, hemolysis, rashes, renal failure, hypertension, bradycardia, heart block, pulmonary edema, cardiac arrest and bronchospasm (Santos *et al.*, 2003). It was reported that intraperitoneal administration of DMSO, at 32 and 62 % (v/v) in saline, affected mice behavior, which was demonstrated via the significantly decreased locomotor activity (Castro *et al.*, 1995). The *in vitro* effect of DMSO in cell culture systems varies depending on the concentrations and cell type (Timm *et al.*, 2013). A wide range of changes in cellular processes and epigenetic alterations in *in vitro* cardiac and hepatic models was observed as a result of exposure to DMSO (Verheijen *et al.*, 2019). Although this discrepancy in DMSO effects may be linked to the variations in the used concentrations and/or tested models, a careful consideration should be taken for every individual application.

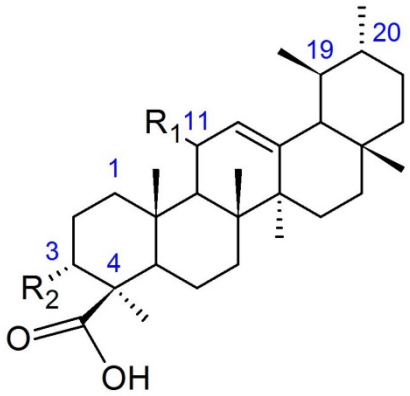
1.2.4 Bioavailability of boswellic acids

Boswellic acids are not an exception of natural products when it comes to limited bioavailability. The level of KBA in plasma was 2 µM following oral intake of 1.6 g *Boswellia* extract, while its EC_{50} to inhibit 5-LO was 2.8 mM (Bagul, Khomane and Bansal, 2014). They also reported that KBA is not stable in stomach and intestine. Gerbeth *et al.* (2011) reported complete absence of AKBA and low concentration of KBA in the serum of 13 out of 14 tested volunteers who had taken four capsules of 400 mg *Boswellia serrata* gum resin extract (BSE) three times per day (daily total was 4800 mg). This could be attributed to poor retention of the extract in the stomach and intestine or to the fact that tested compounds are lipophilic leading to poor absorption, according to the discussed reports. The complete absence of AKBA in plasma could also be linked to metabolism *in vivo*, which will be discussed below.

In a study by Skarke *et al.* (2012), there was an improvement in the level of KBA after eating a standardised meal compared to their level if frankincense was taken in a fasted condition. Moreover, the concentration of KBA was higher than the lower limit of quantitation (LLOQ; <6.66 ng/mL; coefficient of variation <15%) in both conditions fast and fed, which lasted for up to 12h after the intake. This improvement has not been reported for AKBA, but the level of AKBA endured above LLOQ for longer time after the fed condition. Both AKBA and KBA were reported to be bioavailable in plasma, which contradicts the previously discussed

studies. This contradiction might occur for the variations in the used products, physiological status between participants or the experimental design. In addition, AKBA was found in plasma (454 ± 23 ng/mL) and pancreatic tumour tissue (273 ± 13 ng/mL) in orthotopic nude mice 4 h following oral administration of AKBA (Park *et al.*, 2011). AKBA inhibited pancreatic tumour growth and metastasis in the orthotopic nude mouse model of pancreatic cancer and sensitised cells to gemcitabine (Park *et al.*, 2011). Similar observations were reported in rats where the presence of 6 major BAs, including KBA and AKBA, in plasma and brain over the period of 8h following oral administration of BSE was confirmed (Gerbeth *et al.*, 2013).

With regards to metabolism, only a few studies considered the metabolic profiles of BAs despite their use in pre-clinical and clinical studies, in particular for their anti-inflammatory effect, for a relatively long time. It appears that the acetylated BAs, such as AKBA, are metabolically stable while non-acetylated BAs, such as KBA, are prone to extensive phase I metabolism (Krüger *et al.*, 2008; Gerbeth *et al.*, 2013). In a more recent study that investigated the metabolic profile of AKBA and KBA in human preparations *in vitro*, the major metabolic pathway of AKBA was deacetylation to form KBA by carboxylesterase 2, whereas KBA was hydroxylated by CYPs, predominantly by CYP3A4 (Cui *et al.*, 2016). Moderate anti-inflammatory activity was reported for the formed KBA and hydroxylation metabolites compared to their parent compounds (Cui *et al.*, 2016). To our knowledge, there is a lack of evidence if metabolism is required for BAs to exert the reported bioactivity. This indicates to the importance of further investigations of bioefficacy, bioavailability and pharmacokinetics of BSE and individual BAs to develop them as agents in cancer chemoprevention and/or oncotherapy.



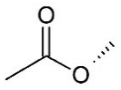
R_1	R_2	Name
-H	-OH	β-boswellic acid (β-BA)
=O		11-keto-β-boswellic acid (KBA)
-H		3-*O*-acetyl-β-boswellic acid (ABA)
=O		3-*O*-acetyl-11-keto-β-boswellic acid (AKBA)

Table 1.4: Chemical structure of BAs

1.3 Drug Combination

A combination of two or more drugs is used to treat various diseases including cancer, as discussed earlier. A number of studies have investigated the possibility of introducing plant extracts as treatments or adjuvants in cancer aiming to either overcome resistance of cancer to chemotherapies or to reduce undesirable side effects of the agents. For instance, the vinca alkaloid vinblastine (extracted from *Catharanthus roseus*) is used in combination with methotrexate, doxorubicin and cisplatin (MVAC) as an alternative therapy protocol for metastasized urothelial cancer. A combination of vinflunine and vinblastine was reported effective against gemcitabine- and cisplatin-resistant urothelial cancer cell lines (Vallo *et al.*, 2015). The synergistic effect between BSE and doxorubicin on human hepatocellular carcinoma was studied and reported by Khan *et al.* (2014).

1.4 Aims of the current project

This project is based on currently unpublished work from Al-Salmani (2017), which indicates that the principal bioactive component of frankincense, AKBA, is able to effectively decreases the viability of ovarian cancer cells *in vitro* using models of high grade serous ovarian cancer and ovarian endometroid adenocarcinoma, which are OVCAR4, A2780, A2780cis and UWB1.289. AKBA cytotoxicity towards tested cell lines was also demonstrated through induction of intrinsic and extrinsic apoptotic pathways, the induction of DNA damage and decreased ROS generation. Moreover, multiple gene expression alterations in the cells were reported following the exposure to AKBA including up-regulation of pro-apoptotic genes and down-regulation of anti-apoptotic genes.

Despite the efficacy of conventional chemotherapies, they are linked to serious side effects and are challenged with the ability of cancer cells to be resistant. This project aims to explore the potential utility and mechanisms of action of AKBA, a bioactive component of frankincense, in overcoming chemoresistance in cancer, using ovarian cancer as the primary model. This is proposed by establishing the cytotoxic effect of extracts of frankincense towards various ovarian cancer cell lines and the ability of the leading bioactive chemical component, AKBA, to synergise with selected chemotherapeutic drugs and show preferential cytotoxicity towards chemoresistant cancer cells.

1.5 The thesis hypothesis

The importance of developing new approaches to treat ovarian cancer is urgent for the discussed reasons above, which are briefly: the late stage diagnosis, the recurrence of the disease, its ability to develop chemoresistance and the undesired side effects of current chemotherapies. Since the toxicity of frankincense extracts and bioactive single components from it, such as AKBA, towards various cancers were reported, it is hypothesised that AKBA will interact synergistically with conventional chemotherapies used to treat ovarian cancer, i.e. cisplatin, doxorubicin and paclitaxel. The interaction between AKBA and selected chemotherapies will be further explored in terms of the possible mechanisms of actions. The other hypothesis is that various extracts of crude frankincense will be toxic towards ovarian cancer as they were to other types of cancers, which was not shown on ovarian cancer before. The proposed plan and the objectives can be found in the flowchart (Figure 1.6).

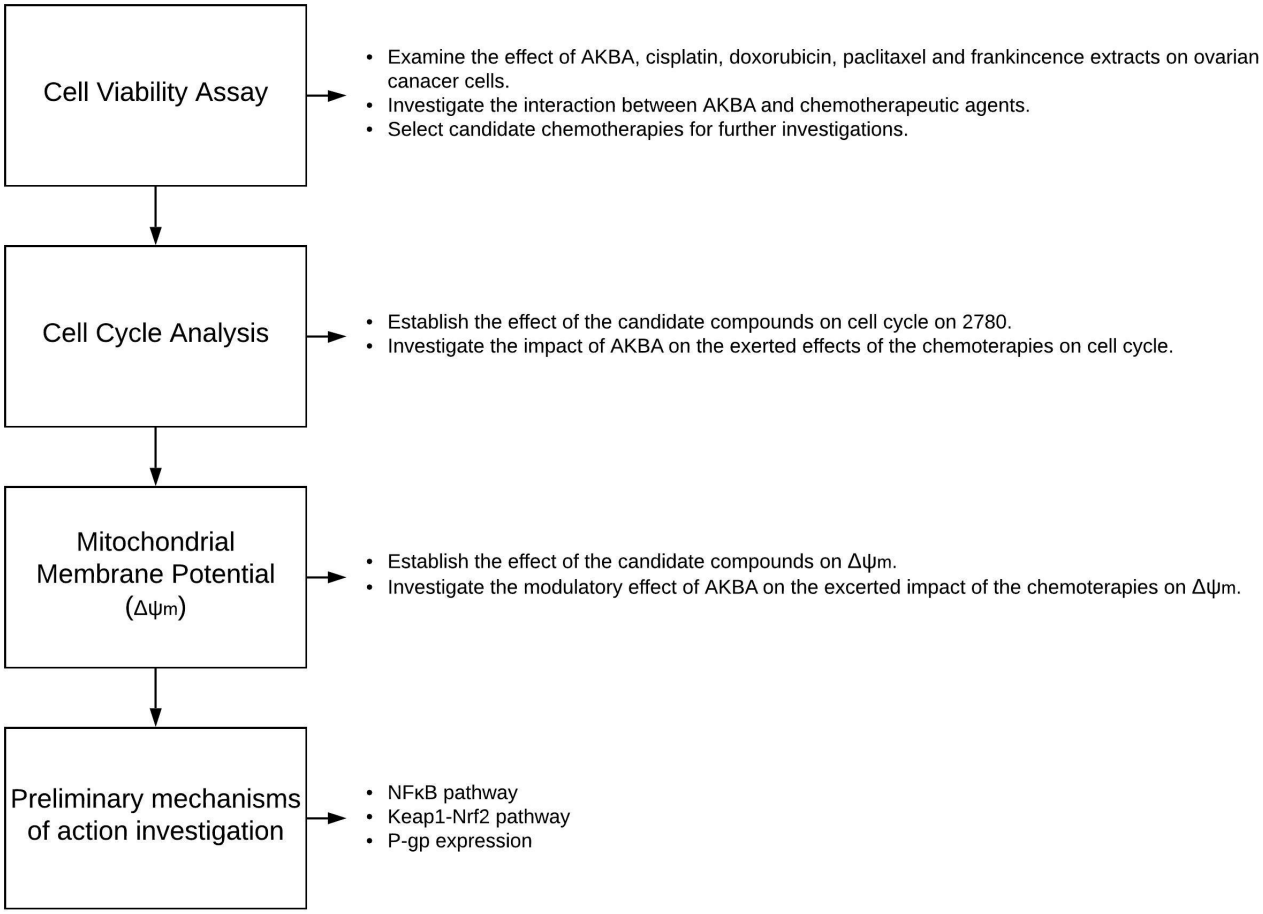


Figure 1.6: A flowchart showing the proposed experiments and the objectives of this project.

Chapter 2

Cell Viability Assessment

2 Cell viability assessment

2.1 Introduction

2.1.1 The therapeutic efficacy testing approaches

This brief introductory section will attempt to briefly address the possible approaches to establish what was discussed above in the introduction of this thesis on cancer and frankincense. 'The development of therapeutics for the clinical management of cancer is traditionally defined in several distinct phases, including discovery, in vitro testing, pre-clinical animal studies, and clinical trials'(Gordon, Brown and Reynolds, 2018). A number of cell-based approaches to determine the therapeutic efficacy were discussed by Gordon et al. (2018). The approaches can be divided into categories as follows. Cell viability and proliferation assays by measuring cell activity through metabolic activity or colony formation assays to evaluate tumour progression *in vitro* as examples. The second category is cytotoxicity assays which can be achieved by staining and imaging, propidium iodide as an example, or lactate dehydrogenase (LDH) release assay. Cell apoptosis assays are the third category that involves DNA fragmentation assays, caspase activation and flow cytometry for the caspases or alive/dead cells stains such as Annexin V and propidium iodide. Finally, cell cycle arrest assays are another approach where the efficacy of anti-tumour agents is tested.

Determination of cellular viability and the morphological changes on the ovarian cancer cells, *in vitro*, that could result from exposing them to the candidate extracts and agents serves as an essential starting point of this project. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay was used to assess cellular viability based on metabolic activity. The following chapters will include cell cycle analysis and investigations of possible molecular targets of the agents.

2.1.2 *In vitro* models of human ovarian cancer

Considering the above, ovarian cancer has been shown to be complex and distinct with multiple sub-types based on histological and pathological categorisation. The multiplicity is also apparent on molecular levels. It is then critical to utilise appropriate *in vitro* models in pre-clinical studies. The publicly available ovarian cancer cell lines are about 100 with various cellular and molecular features (Beaufort *et al.*, 2014). Since EOC cancer represents

90 % of all ovarian cancers (Gloss and Samimi, 2014), it is justifiable to consider using EOC *in vitro* models in this project which will be mentioned below (see 2.1.2.1 an 2.1.2.2).

2.1.2.1 A2780, A2780cis and A2780ADR

A2780 is an epithelial ovarian cancer cell line that was derived from an ovarian endometroid adenocarcinoma tumour in an untreated patient (Public Health England, 2016). A2780 is one of the most commonly used *in vitro* models of ovarian cancers which accounts for 60 % of the publications with SK-OV-3 cell line. In recent years, the use of A2780 as model of HGSOC is being questioned due to the lack of the key genomic characteristic of HGSOC which include *TP53* mutations and substantial copy-number changes (Anglesio *et al.*, 2013; Domcke *et al.*, 2013; Bourgeois *et al.*, 2015; Tudrej *et al.*, 2018). It is also mentioned that A2780 is most likely part of an endometroid histological sub-type (Beaufort *et al.*, 2014).

In an attempt to understand the mechanisms of chemoresistance in ovarian cancer and to investigate possible approaches to overcome it, researchers developed resistant variants of A2780 called A2780cis and A2780ADR. The resistance to cisplatin, A2780cis, and to doxorubicin, A2780ADR, was induced by the chronic exposure of the parent A2780 cell line to the chemotherapies (Behrens *et al.*, 1987; Public Health England, 2013b, 2013a). These developed variants serve as a crucial tool in cancer research.

2.1.2.2 OVCAR4

Taking into account the potential lack of resemblance of A2780 to HGSOC, the cytotoxicity of various treatment approaches were also examined on OVCAR4. OVCAR4 shares with A2780 the fact that the histological subtype was not attributed by the originator. However, OVCAR4 is widely accepted as a model of HGSOC based on its genomic features (Domcke *et al.*, 2013). In addition to the mutation in TP53, Bourgeois *et al.* (2015) demonstrate a number of molecular features that OVCAR4 shares with ovarian tumours such as the expression of matrix-metalloproteinase-2 (MMP2) which is found to be increased in ascites fluid in advanced stage ovarian cancer and is associated with the ability of tumour to invade surrounding tissues. OVCAR4 was derived from patient who was previously treated with cyclophosphamide, cisplatin and doxorubicin and became resistant to the treatment (Louie *et al.*, 1985).

Considering the above discussed details on the cell lines, A2780 is an appropriate candidate model as sensitive cell line without previous history of exposures to the tested agents and the possible development of and comparison to the resistant variants, A2780cis and A2780ADR. In addition, OVCAR4 is another superior model to confirm the effects of the tested agents on ovarian cancer due to the discussed higher relativity to ovarian cancer in patients.

2.1.2.3 Cell culture limitations

Researchers use patient biopsies, genetically engineered mouse models, patient-derived xenografts, patient stem cell-derived organoids and/or cancer cell lines to study human cancer cell biology. Despite the acknowledged lack of human biology recapitulation, cell lines remain the most commonly used system for being practical and relatively easy to handle (Hynds *et al.*, 2018). A human cancer cell line only represents a snapshot of a tumorigenic process, which consists of multiple stages in humans, and a single type of cells whereas tumour microenvironment consists of various cell types (Hynds *et al.*, 2018). The other key limitation of cancer cell culture is heterogeneity and genetic instability, where cells continue to evolve in culture leading, perhaps, to discrepancy in the results (Ben-David *et al.*, 2018). The heterogeneity may, however, be used as an advantage (Hynds *et al.*, 2018) since genomic instability is one of the hallmarks of cancer (Hanahan and Weinberg, 2000, 2011).

Other differences are related to *in vivo* and *in vitro* culture conditions, where *in vivo* conditions are physiologically controlled and *in vitro* conditions are artificially optimised. The concentration of serum, for instance, in solid tumours is less than 1 %, while the concentration range in cell culture is 3-10 % (Staveren *et al.*, 2009). Moreover, the atmospheric oxygen concentration in standard cell culture incubators is about 20 % compared to 2-9 % physiological level in tissues, which is even lower in tumours (Bertout, *et al.*, 2008; Staveren *et al.*, 2009; Geraghty *et al.*, 2014). It is noteworthy to mention that the high atmospheric oxygen level does not necessary reflect the pericellular concentration of oxygen. The condition may vary from hyperoxia to near-anoxic due to oxygen's inert diffusion rate through aqueous medium, which is affected by cell density, media volume and barometric pressure (Place, Domann and Case, 2017).

This brief overview covered some of the differences between *in vivo* and human cancer cell culture and indicate to the importance of monitoring culture conditions in order to increase data reproducibility and reliability. It is, as well, crucial to continue to develop cell culture

models to improve their relevance to human tumours. Cancer cell lines are still one of the most useful experimental systems in modern cancer research and shares the following hallmarks of cancer with cancer cells *in vivo*: self-sufficiency in growth control, insensitivity to antigrowth signals, escape from checkpoints, immortality, genetic instability, loss of negative feedbacks and invasiveness (Staveren *et al.*, 2009; Place, Domann and Case, 2017). Finally, the discussed points should be taken into consideration when interpreting results on cell culture and more importantly data should not be extrapolated to *in vivo* models without proper testing.

2.2 Materials and Methods

2.2.1 Materials

All materials were obtained from Thermo Fisher Scientific (Loughborough, UK) or Sigma-Aldrich via SLS (Huddersfield, UK), unless noted otherwise.

- RPMI-1640 medium
- Phosphate buffered saline (PBS)
- Newborn calf serum (NCS)
- Dimethyl sulfoxide (DMSO)
- Trypsin-EDTA (Ethylenediaminetetraacetic Acid)
- Ethanol
- Trypan blue
- Cell culture plastic wares
- 96-well microplate
- MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)
- Haemocytometer (cell count chamber)
- Freezing box

2.2.2 Compounds and drugs

3-Acetyl-11-keto-β-boswellic acid (AKBA, solvent: DMSO), Cisplatin (cis-Diamineplatinum(II) dichloride, solvent: PBS), doxorubicin hydrochloride (solvent: DMSO) and Paclitaxel (solvent: DMSO) were purchased from Sigma-Aldrich (St Louis, MO, USA). PBS was used to dissolve cisplatin due to the structural and functional changes that could arise from dissolving platinum based compounds using DMSO (Hall *et al.*, 2014). AKBA was also supplied by Dr A. Al-Harrasi, University of Nizwa, Oman, which was only used in the beginning of this project on A2780 for MTT assay. The difference in EC_{50} was negligible (EC_{50}= 30.76 µM, n=3, AKBA from Dr A. Al-Harrasi) (EC_{50}= 32.58 µM, n=1, AKBA from Sigma).

2.2.3 Cell lines and culture

The A2780 cell line is derived from an ovarian endometroid adenocarcinoma tumour in an untreated patient, the cisplatin resistant counterpart A2780cis and the doxorubicin resistant counterpart A2780ADR were purchased from Sigma-Aldrich (St Louis, MO, USA). The vials of cells contained 1 mL of cells and were frozen in liquid nitrogen. To revive, cells were thawed at 37 °C, transferred from a cryovial to a 15 mL screw cap, conical polypropylene tube and 9 mL RPMI-1640 medium, plus GlutaMAX^{TM} (2 mM) and 10 % v/v NCS washed with RPMI-1640 medium and then PBS, and transferred to fresh medium in a T75 (surface is 75 cm^2) flask to be cultured and maintained. After 24 hours, medium was replaced with fresh medium. Cells were maintained in RPMI-1640, plus 2 mM GlutaMAX^{TM} and 10 % v/v NCS at 37 °C in a humidified atmosphere of 5 % CO_2 to be sub cultured every 48 hours, when the confluence was between 70 and 80 %. In addition, 1 μM cisplatin was added to the A2780cis cell line and 100 nM doxorubicin to the A2780ADR cell line every two to three sub-cultures to maintain resistance. It is noteworthy to mention that a passage number limit was introduced based on the observed change in the growth rate for each cell line. For example, A2780 cells were used in experiments between revival (passage 13) and passage 30, where changes in the growth pattern usually happen. Similar range was applied for A2780cis and A2780ADR, while the range was between passages 14 and 18 for OVCAR4.

The other cell line used in this project was OVCAR4 cell line that was a kind gift from Professor Iain McNeish, Beatson Institute of Cancer Sciences, University of Glasgow, UK. OVCAR4 cells were originally obtained from Dr R. Camalier, Developmental Therapeutics Program, National Cancer Institute MD, USA. OVCAR4 is a high grade serous adenocarcinoma from ovarian epithelium which was derived from a patient resistant to platinum-based chemotherapy (Lengyel *et al.*, 2014). The Following conditions were followed to grow and maintain OVCAR4 cells: growth medium was DMEM (Dulbecco's Modified Eagle Medium) + 2 mM GlutaMAX^{TM} + 4.5 g/L D-glucose + 110 mg/L pyruvate and incubated at 37 °C in a humidified atmosphere of 5 % CO_2.

To sub culture and seed, the medium was removed and cells washed with PBS at 70-80 % confluency, which then followed by trypsinisation, trypsin-EDTA was used to detach cells from the surface. The split ratio of sub cultured cells was between 1:2 and 1:6 following the provider's recommendation, which entirely depended on cell proliferation. The effect of trypsin-EDTA was neutralised using culture medium plus NCS. Enough volume of fresh

medium was added to the newly seeded cells to be incubated for further uses. For example, 6 to 9 mL of fresh medium is added to a T25 flask that will be incubated for 48 to 72 h before the next sub culture.

To freeze vials of the cells, cells were sub-cultured in T75 flasks to produce sufficient numbers of cells for freezing, *e.g.* 10 flasks with cells at 80 % confluence gave approximately 100 million cells in total of A2780 cells. Detached cells were combined and pelleted at 400 xg, 4 minutes in a centrifuge and a suitable volume of freezing medium (RPMI-1640 + 2mM Glutamine + 10% v/v NCS + 8 % v/v DMSO) was added to the pellet to give a cell density of 3.5 x 10^6 cells per mL. Aliquots of 1 mL of this suspension was divided to an appropriate number of cryovials. The vials were transferred to a freezing container and kept at – 80 °C overnight, then transferred to liquid nitrogen for longer term storage.

2.2.4 Seeding and treatment

In order to seed cells for treatment, cells were counted in a haemocytometer to determine the approximate density of the cell suspension following trypsinisation, this was then used to calculate the volume of cells to be added to the wells of a sterile 96 well plate to get the appropriate starting density per well (see section: 2.3.1). Cells were seeded in a 96-well microplate and then incubated for 24 hours to adhere to the plate, grow and recover from the seeding. After 24 hours cells were treated with various concentrations of the desired compound for 24 hours to be used in cytotoxicity assays (see below). A number of attempts were required to reach an optimal concentration range to establish the EC_{50} of a compound that had not been well investigated. For instance, various concentrations of paclitaxel were used starting from a very low concentration to a very high concentration, wide dose ranges which were then used to refine the dose range for determining EC_{50}.

The combination of AKBA with tested drugs was performed as follows: cells were treated with 26 μM of AKBA (See section: 2.3.3), which was lower than the EC_{50} of AKBA on all cell lines, and various concentrations of the agent simultaneously for 24 hours. The effect of the combination on cells was measured using a viability assay (see below).

The other approach of treatment was as follow. Cells were pre-exposed to 26 μM AKBA alone for 24 hours. Then, AKBA was removed and replaced with a fresh medium and various concentrations of doxorubicin. Finally, MTT assay was performed. This approach for some

exposure experiments was taken to examine if the effect of AKBA, for example gene expression, signalling or metabolic effects, persisted after the AKBA had been removed.

2.2.5 Frankincense extraction

5.2 g of Omani frankincense resin (al hojary grade) was ground to a fine powder using a pestle and mortar. 4.95 g of the fine powder was transferred to a fresh tube, where 20 mL of 70 % v/v ethanol in water was added. The slurry was left on an orbital shaker overnight. Then, the mixture was centrifuged at high speed to remove particles. The resultant material comprised of three layers: precipitated particles (at the bottom), oily phase (in the middle) and clear liquid phase (at the top). The oily and clear phases were transferred together to a fresh 15 mL screw top polypropylene tube and then were centrifuged again to remove any remaining particles. The two layers were separately transferred to two clean 50 mL round bottomed flasks and the solvent was removed using a rotary evaporator with water bath set at 50 °C. Residual water was removed by freeze drying for 7- 8 h until the weight of the flask stabilised. The dry extracts were stored at -20 °C until further use. Solvent-free extracts were reconstituted in DMSO.

To mimic the traditional use of the frankincense resin, two pieces of roughly equivalent size resin were put in distilled water for 24 hours. A similar procedure to that was used for the 70 % v/v ethanol extracts was applied to the water extract (only freeze drying was used) excluding dissolving the dried product in DMSO. The final dry product was dissolved in sterile PBS and filter sterilised using a 0.22 µm syringe filter.

2.2.6 Cell viability assay

Cell growth/viability was determined by the 3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay. The principal mechanism of the assay relies on the production of intracellular purple formazan by metabolically active cells as a metabolic product of reduced tetrazolium salts, which can be then solubilised and quantified using spectrophotometer. The treatment medium from the treatment (see above) was replaced with 200 µL fresh medium. A final concentration of 0.5 mg/mL of MTT solution (stock solution: 5 mg/mL MTT in PBS) was added to the cells in fresh medium and

incubated for 2 hours at 37 °C in a humidified atmosphere of 5 % CO_2. Medium with MTT was removed and crystals then dissolved using 150 µL DMSO, which was then incubated for 15 minutes at 37 °C in 5 % CO_2. An inverted light microscope was used to confirm that crystals were totally dissolved. The absorbance was measured using a microtitre plate reader (GloMax® Discover System, GM3000, Promega Corporation, USA) at 600 nm. Data are presented as graphs showing the mean and SEM plots of three independent experiments (See section: 2.2.8).

To determine the optimal number of cells for seeding for MTT assay (for subsequent viability assay), various numbers of cells were initially added per well, covering the range 10,000 to 80,000 cells per well. Cells were incubated for 48 hours, medium was replaced with fresh medium after the first 24 hours. The optical density was read on the microtiter plate reader at a wavelength of 600 nm. The optimal cellular seeding number was determined using the data from three independent experiments. Graphs were plotted using Microsoft Excel (for Mac, version 15.28). The optimal seeding density was determined based on a seeding density that gave a final absorbance reading of 0.75 - 1.25 (American Type Culture Collection, 2011).

2.2.7 Isobolographic analysis

The interaction between AKBA and each one of the selected chemotherapeutic drugs, cisplatin, doxorubicin or paclitaxel, was evaluated by treating cells with different concentrations of one drug in the presence or absence of AKBA. The EC_{50} was determined by plotting data of three independent experiments. The combination index (CI) was used to determine the interaction by applying the following formula:

$$CI = \frac{D1}{DX1} + \frac{D2}{DX2} + \frac{(D1 \times D2)}{(DX1 \times DX2)}$$

where $Dx1$ is dose of drug 1 to produce 50 percent cell viability alone; $D1$ is dose of drug 1 to produce 50 percent cell viability in combination with $D2$; $Dx2$ is dose of drug 2 to produce 50 percent cell viability alone; $D2$ is dose of drug 2 to produce 50 percent cell viability in combination with $D1$ (Chou and Talalay, 1983; Hossain *et al.*, 2012; Khan *et al.*, 2014).

2.2.8 Statistical analysis

The EC_{50} was determined using nonlinear regression (curve fit) on GraphPad Prism (version 7.0b for Mac OS X, GraphPad Software, La Jolla California USA, www.graphpad.com). Results are shown as mean ± SEM. Since the mean of the means of each dose of 3 independent experiments is shown, SEM was chosen to show the difference between the means and how precise the means are (Hertzog, 2017). Unpaired t-tests using GraphPad Prism (version 7.0b for Mac OS X, GraphPad Software, La Jolla California USA, www.graphpad.com) were used to assess if the difference between the effect of each compound on the examined cell lines was statistically significant. This was also applied to test the significance of the difference between the level of cytotoxicity of the investigated compounds and the effect of the combination by comparing the EC_{50} values or the viability of the cells on every single dose. The difference was considered statistically different, if $P<0.05$.

2.3 Results

2.3.1 Experiment optimisation

2.3.1.1 Optimal cell seeding number for MTT assay

As an example, the optimal seeding number for A2780 lies between 25,000 and 35,000 cells per well at the wavelength of 600 nm (Figure 2.1). However, in subsequent experiments it was noted that the optimal seeding number depends on the growth rate of the cells proliferating prior to seeding. For example, 20,000 cells per well is the optimal density if A2780 cell count is more than 3 million cells/mL when the contents of a T25 flask are harvested, so seeding number was adjusted accordingly. Optimal seeding number for other cell lines used was determined individually as appropriate.

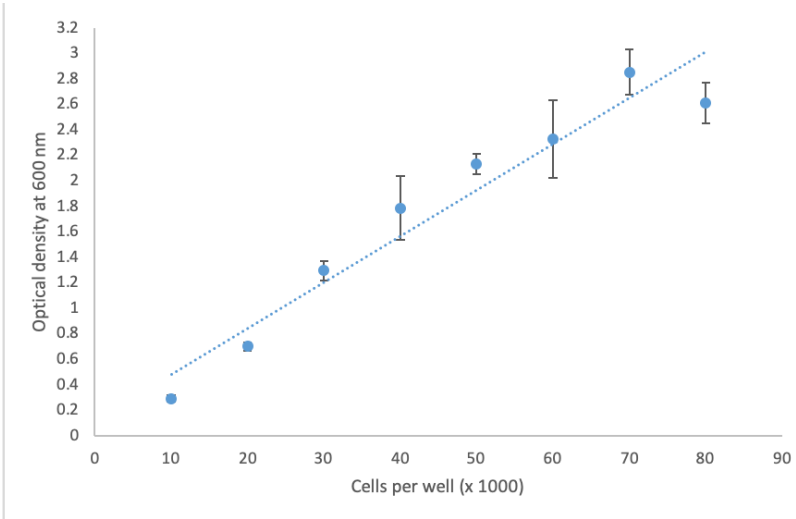


Figure 2.1: Optimal cell seeding number for MTT assay of A2780 at 600 nm. Data are shown as mean ± SEM for n= 3 independent experiments.

2.3.1.2 Assessing DMSO toxicity

The other part of optimisation considered the percentage of DMSO per well. A broad range of DMSO percentages was used to deliver various concentrations of dissolved AKBA, doxorubicin or paclitaxel to the cells. The highest percentage of DMSO in a well was 0.74 % v/v in medium. A concentration of 0.8 % v/v DMSO in medium was monitored regularly to confirm that DMSO was not affecting the viability of the cells. For example, the viability of A2780 cells exposed to 0.8 % v/v DMSO in medium was 97 ± 2 % (n=3) of the negative control. The difference between the viability of negative (untreated cells) control and cells exposed to DMSO was not statistically significant (P=0.17).

2.3.2 In vitro cytotoxicity assay

2.3.2.1 The level of cytotoxicity of the tested agents individually on the selected cell lines

The cytotoxicity of AKBA, cisplatin, doxorubicin, paclitaxel and the two parts of 70 % v/v ethanol frankincense extract (clear, FEc, and oily, FEo) on all or some of the selected cell lines, A2780, A2780cis, A2780ADR and OVCAR4, was assessed *in vitro* using the MTT assay. A cell line is considered as a resistant cell line if the EC_{50} of resistant cells/EC_{50} of parental cell is more than 2, called resistance factor (Vallo *et al.*, 2015). The EC_{50} values for AKBA, cisplatin, doxorubicin, paclitaxel, FEc and FEo on A2780 were 30.76 ± 1.71 μM, 12.36 ± 2.15 μM, 1.96 ± 0.33 μM, 80 ± 9.5 nM, 14 ± 2 μg/mL and 30.46 ± 2.3 μg/mL, respectively (Figure 2.2).

A2780cis was resistant to cisplatin as the EC_{50} value was 38.62 ± 7.3 μM, where the resistance factor (RF) is 3.12. In contrast, A2780cis cells were as sensitive to AKBA (EC_{50} 35.64 ± 0.78 μM), doxorubicin (0.97 ± 0.19 μM), paclitaxel (7 ± 1.46 nM) , FEc (11.5 ± 3 μg/mL) and FEo (32 ± 5.5 μg/mL) as A2780 (Figure 2.3). The difference between the effect of AKBA on A2780 and A2780cis was not statistically significant (P>0.05), whereas the difference between the EC_{50} of both cisplatin and doxorubicin on A2780 and A2780cis was statistically significant (P<0.05).

The EC_{50} value of doxorubicin on A2780ADR (49 ± 5 μM, RF = 25) shows how resistant the cell line was to doxorubicin compared to its sensitive parent, A2780. Additionally, the EC_{50} value of AKBA on A2780ADR was 30 ± 2 μM, which is similar to EC_{50} on A2780 (Figure 2.4). The difference between the EC_{50} of doxorubicin on A2780ADR compared to its EC_{50}s

on A2780 A2780cis and OVCAR4 was statistically significant ($P<0.0001$), while the EC_{50} of AKBA on A2780ADR was not significantly different to the other cell lines.

The tested candidate agents were also toxic to OVCAR4 with the following EC_{50} values 40 ± 4 μM for AKBA, 8 ± 2 μM for cisplatin and 2.4 ± 0.3 μM for doxorubicin (Figure 2.5). The difference between the EC_{50} of cisplatin or doxorubicin on OVCAR4 compared to the other cell lines was only statistically significant once compared to the resistant cell lines that are A2780cis for cisplatin ($P<0.01$) and A2780ADR for doxorubicin ($P<0.0001$).

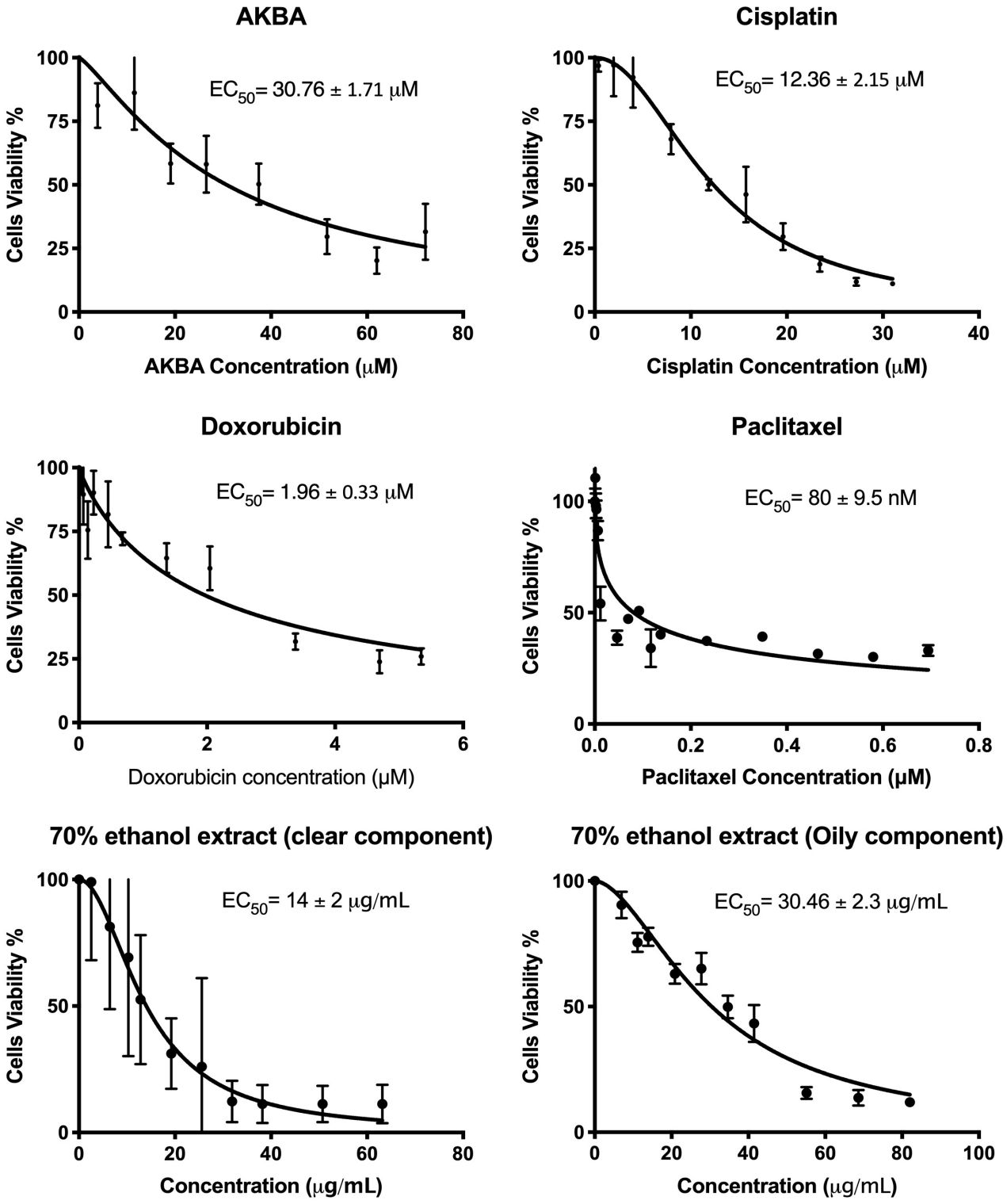


Figure 2.2: Cytotoxicity of AKBA, cisplatin, doxorubicin, paclitaxel and both the clear and oily components of the 70 % v/v ethanol frankincense extract on A2780 cells. *The mean ± SEM of at least n = 3 independent experiments is shown.*

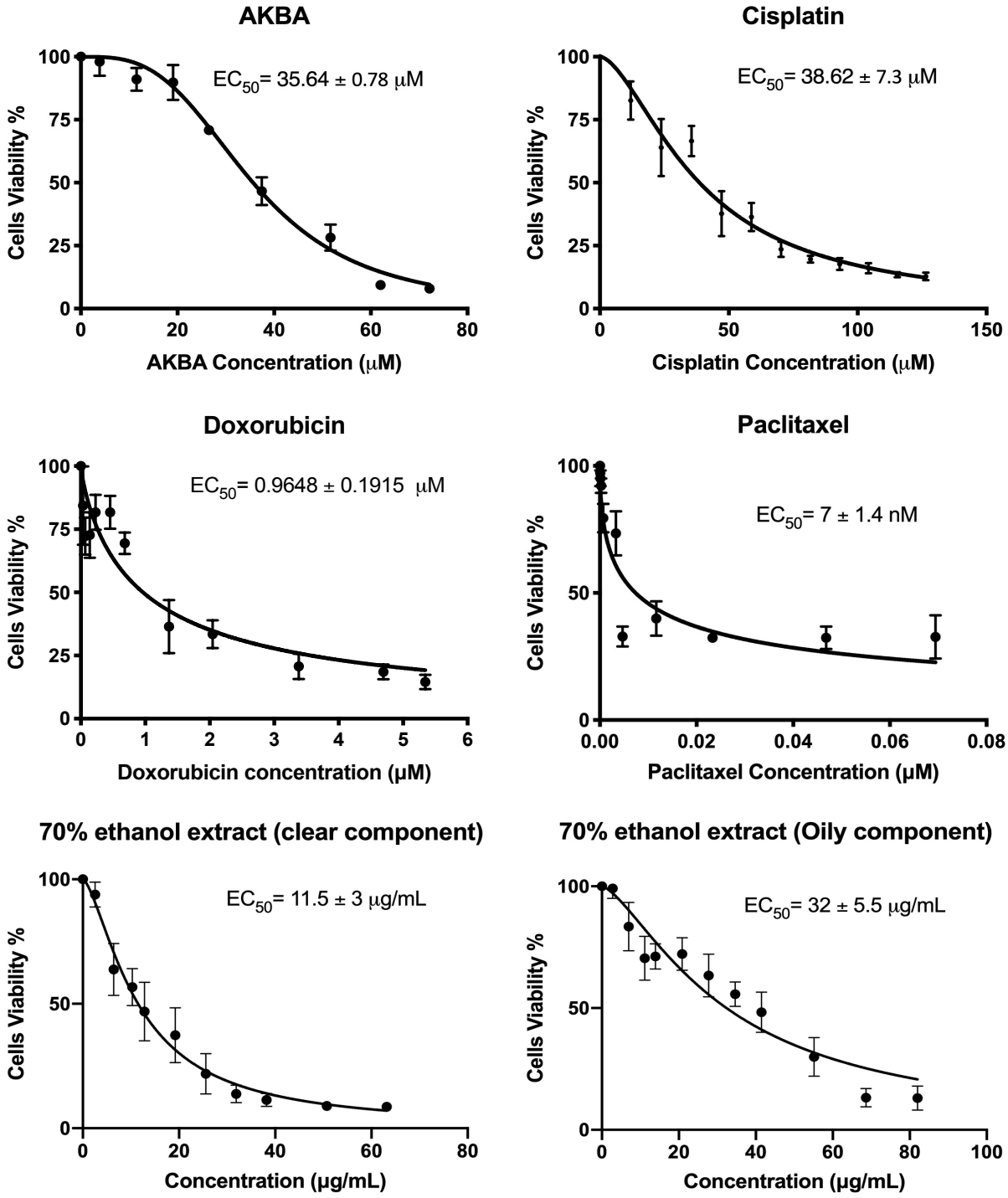


Figure 2.3: Cytotoxicity of AKBA, cisplatin, doxorubicin, paclitaxel and both the clear and oily components of the 70 % v/v ethanol frankincense extract on A2780cis cells. *The mean ± SEM of at least n = 3 independent experiments is shown.*

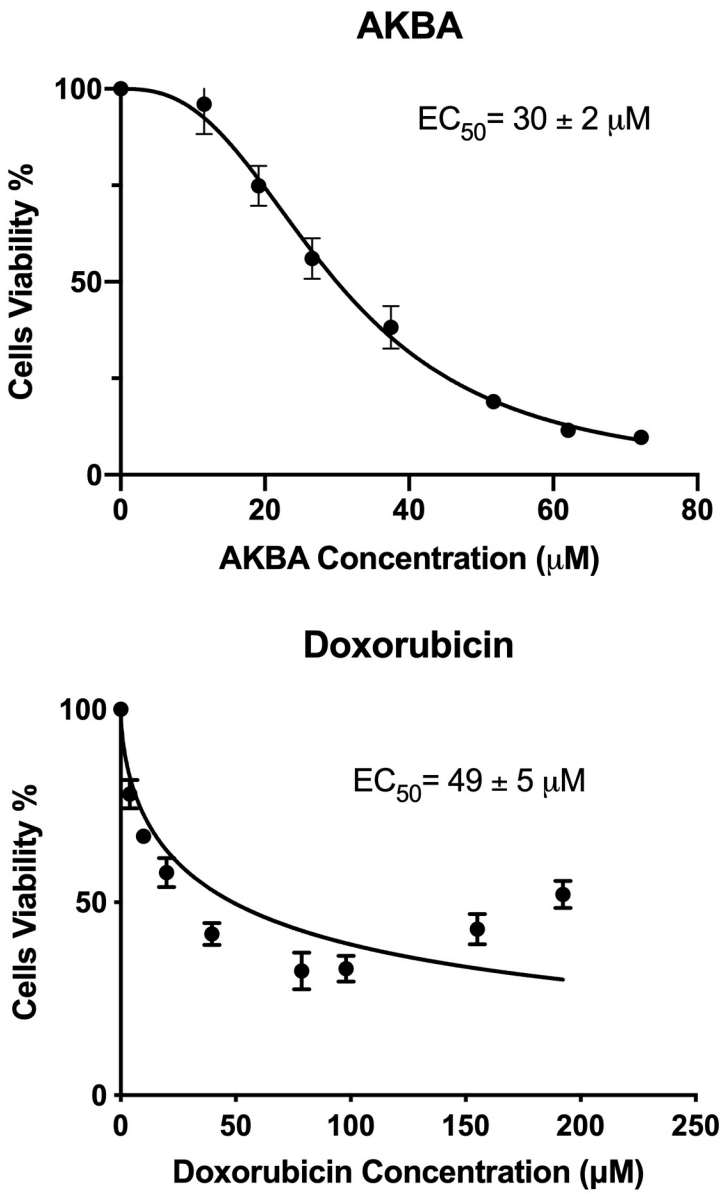


Figure 2.4: Cytotoxicity of AKBA and doxorubicin on A2780ADR cells. *The mean ± SEM of at least n = 3 independent experiments is shown.*

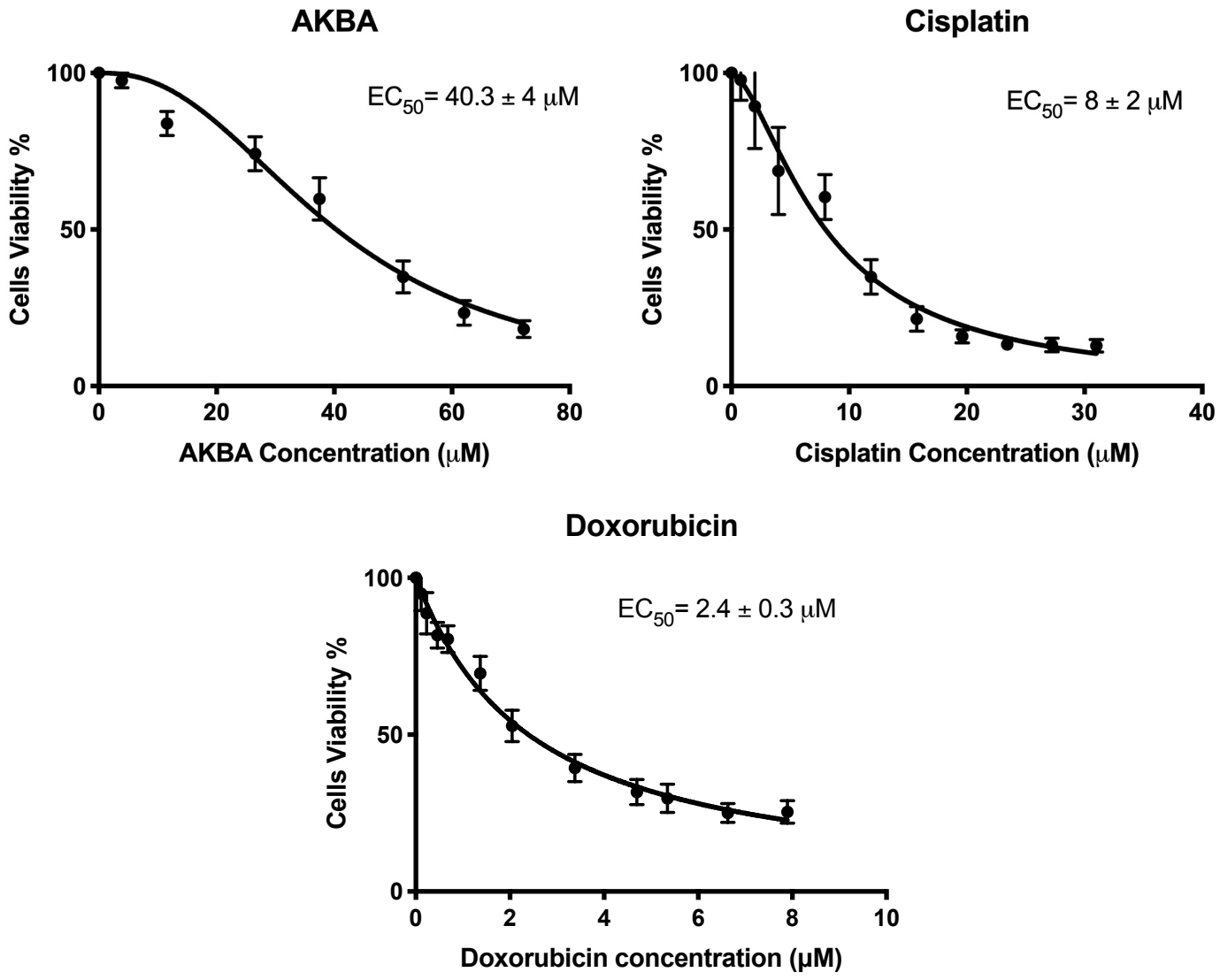


Figure 2.5: Cytotoxicity of AKBA, cisplatin and doxorubicin on OVCAR4 cells*. The mean ± SEM of at least n = 3 independent experiments is shown.*

2.3.3 The interaction of AKBA with tested chemotherapies

2.3.3.1 Cisplatin with AKBA

The results of treating A2780 cell line with various concentrations of cisplatin and 26 μM of AKBA simultaneously for 24 hours in three independent experiments indicated that there was antagonistic effect between AKBA and cisplatin at doses higher than the EC_{50} of cisplatin on A2780 as shown in the graph (Figure 2.6) and confirmed after calculating the CI value (Table 2.1). Data on A2780cis confirms the antagonism between AKBA and cisplatin including the low doses of cisplatin (Figure 2.6 and Table 2.1). The EC_{50} of both methods of treatment was not statistically different from the EC_{50} of cisplatin alone ($P>0.05$). Comparing the EC_{50} simply does not reflect the apparent negative effect of the presence of AKBA with cisplatin at doses above the EC_{50}. Therefore, another statistical approach was applied by comparing single doses of cisplatin in the presence and absence of AKBA. This revealed that the antagonistic effect of AKBA on cisplatin on both cell lines was statistically significant when cisplatin dose is more than the EC_{50}. Finally, moderate antagonism was found between AKBA and cisplatin on OVCAR4 (CI=1.3) even though the EC_{50} was, to some degree, improved, where the EC_{50} was halved in the presence of AKBA compared to the EC_{50} when cells were exposed to cisplatin alone (Figure 2.6). Despite the CI value, the interaction between AKBA and cisplatin on OVCAR4 is negligible.

2.3.3.2 Doxorubicin-AKBA interaction

The interaction between doxorubicin and AKBA was assessed by treating cells with various concentrations of doxorubicin and 26 μM of AKBA simultaneously for 24 hours. The EC_{50} of the combination was 29, 23, 25 and 16 fold less than the EC_{50} of treating cells with doxorubicin alone on A2780, A2780cis, A2780ADR and OVCAR4 cells, respectively (Figure 2.7). There was a synergistic effect between AKBA and doxorubicin on A2780, A2780cis, A2780ADR (pre-exposed to AKBA) and OVCAR4 and an additive action on A2780ADR (AKBA added simultaneously with doxorubicin) (Table 2.1). These effects were statistically significant ($P<0.05$).

Table 2.1: Combination index (CI) results from isobolographic analysis

Treatment	CI			
	A2780	A2780cis	OVCAR4	A2780adr
Cisplatin + AKBA	3	3.2	1.3	-
Doxorubicin + AKBA	0.9	0.81	0.75	0.94/pre-exposure 0.87
Paclitaxel + AKBA	1.09	1.36	-	-

CI = or greater than 1.3: antagonism; CI = 1.1– 1.3: moderate antagonism; CI = 0.91–1.1: additive effect; CI = 0.81–0.9: slight synergism; CI = 0.61–0.8: moderate synergism; CI = 0.41–0.6: synergism; and CI = 0.2– 0.4: strong synergism. Data represents the mean of three (n=3) independent experiments. (Aghi et al., 2006; Khan et al., 2014).

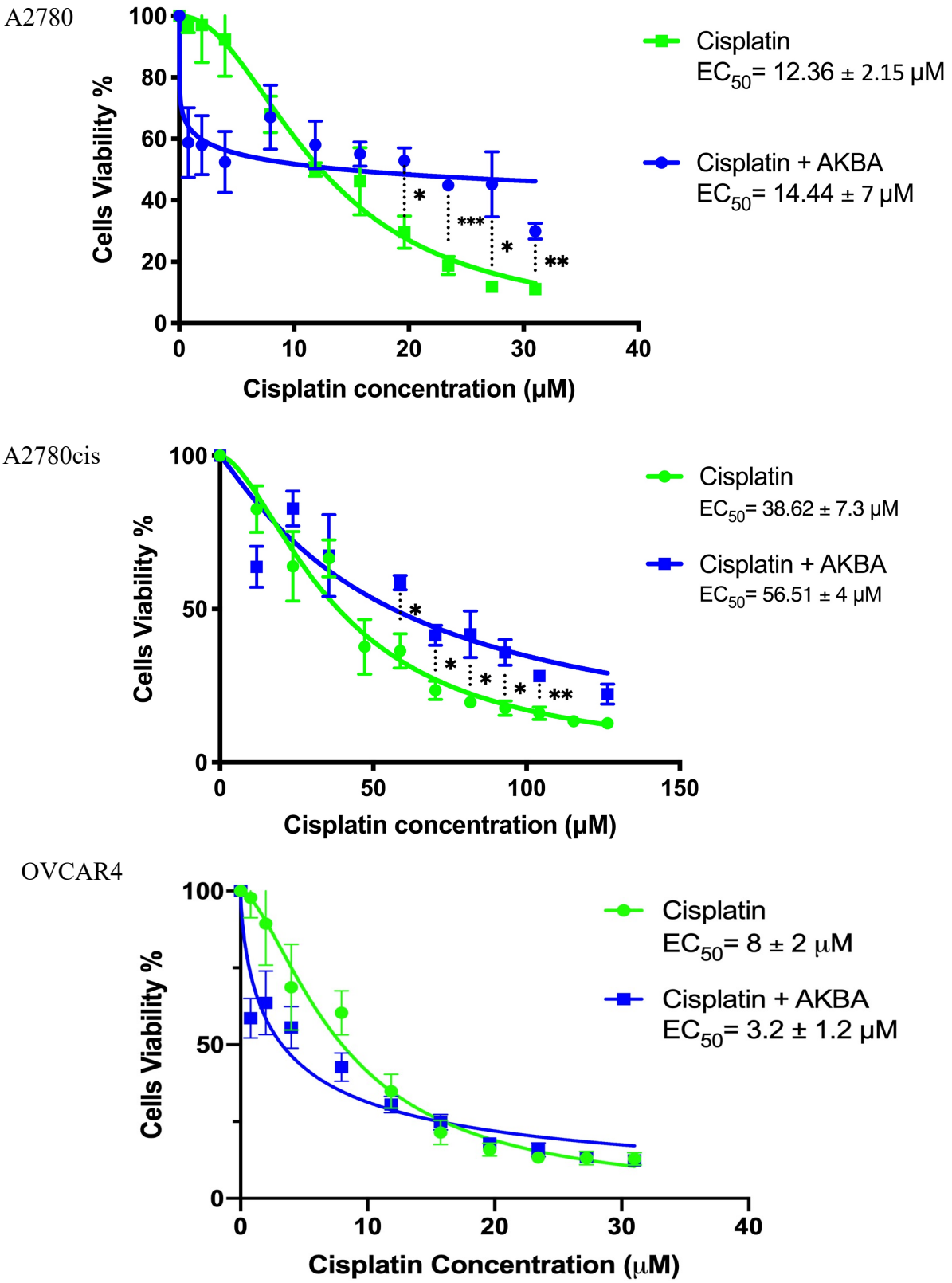


Figure 2.6: Cytotoxicity of cisplatin on A2780, A2780cis and OVCAR4 cell lines treated with and without AKBA*. Cells were treated with 26 µM AKBA and various concentrations of cisplatin for 24 hours simultaneously to test the synergism between these compounds (blue). Cells were treated with cisplatin alone (green). The mean ± SEM for n = 3 independent experiments is shown. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.*

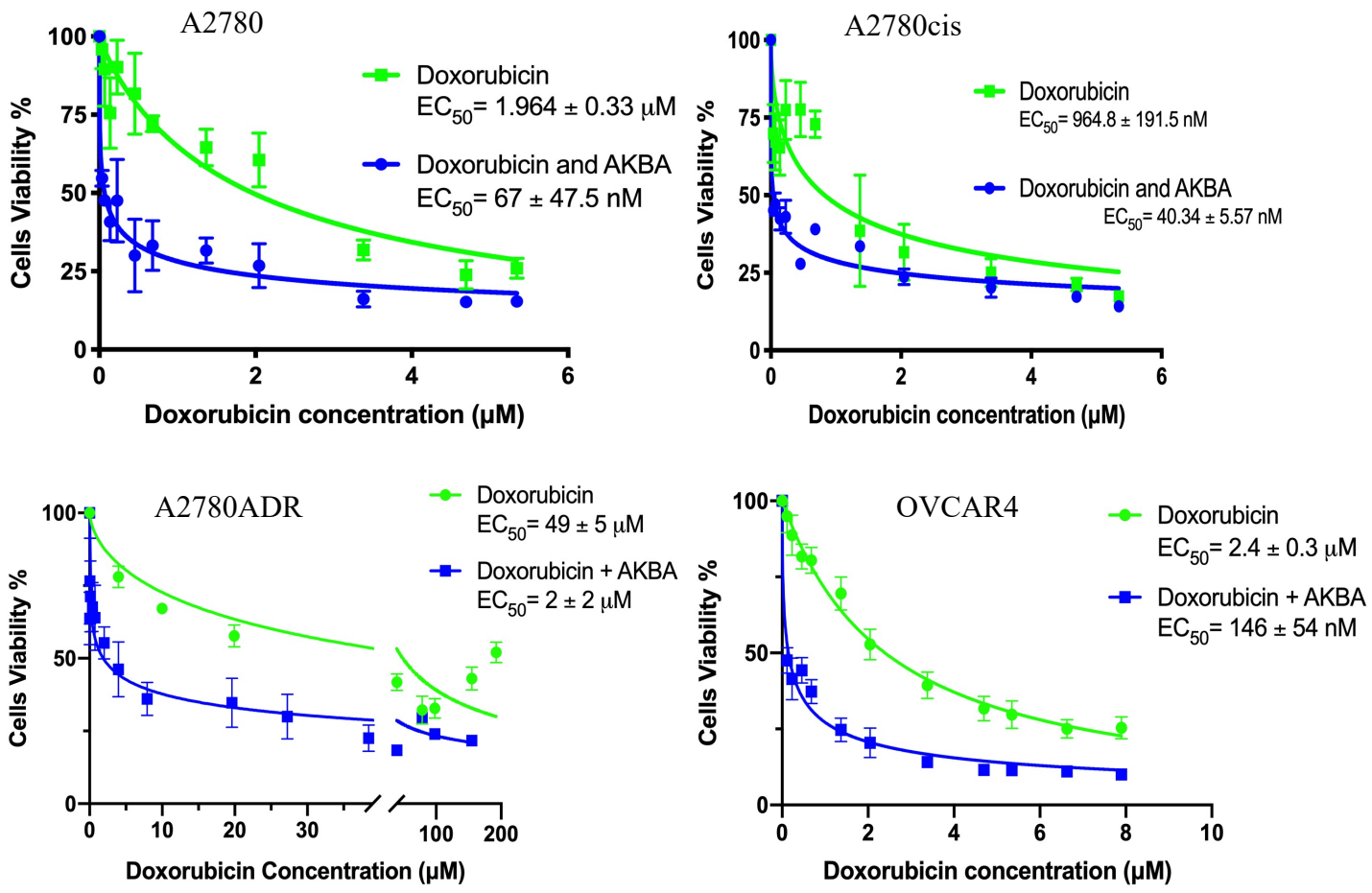


Figure 2.7: Cytotoxicity of doxorubicin on A2780, A2780cis, A2780ADR and OVCAR4 cell lines treated with and without AKBA*. Cells were treated with 26 µM AKBA and various concentrations of doxorubicin for 24 hours simultaneously to test the synergism between these compounds (blue). Cells were treated with doxorubicin alone (green). The mean ± SEM for n = 3 independent experiments is shown.*

2.3.3.3 Paclitaxel with AKBA

Paclitaxel was used alone and in the presence of 26 µM AKBA on A2780 and A2780cis cells. The EC_{50} was slightly lower on both cell lines when the cells were exposed to both paclitaxel and AKBA (Figure 2.8). However, the CI value showed that there was additive effect (on A2780) and moderate antagonism (on A2780cis) between AKBA and paclitaxel (Table 2.1).

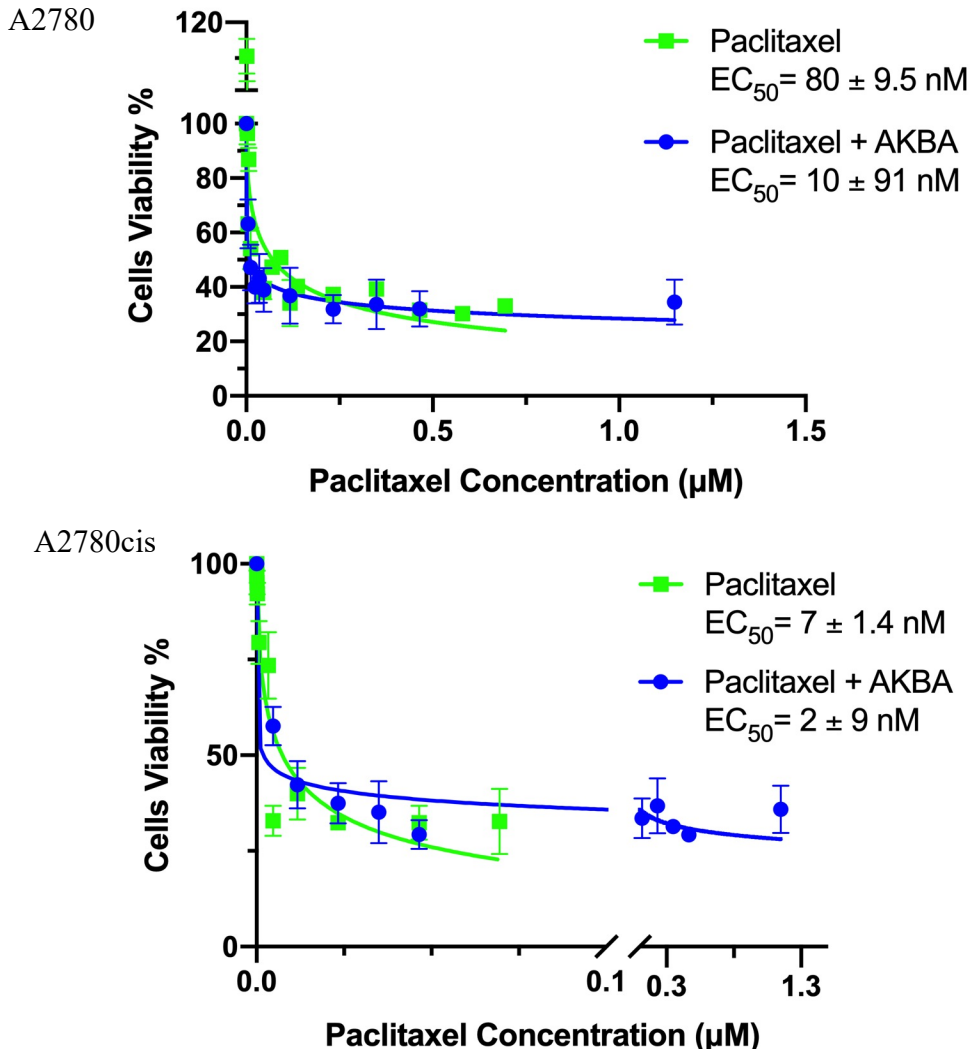


Figure 2.8: Cytotoxicity of paclitaxel on A2780 and A2780cis cell lines treated with and without AKBA. *Cells were treated with 26 µM AKBA and various concentrations of paclitaxel for 24 hours simultaneously to test the synergism between these compounds (blue). Cells were treated with paclitaxel alone (green). The mean ± SEM for n = 3 independent experiments is shown.*

2.3.3.4 Modulation doxorubicin cytotoxicity by pre-exposure of A2780 cells to AKBA

A2780ADR cells were exposed to 26 µM of AKBA for 24 h then the medium was replaced with fresh medium containing various concentrations of doxorubicin and cells exposed to doxorubicin for 24 h. The reason for using this approach was to examine that the apparent synergism between AKBA and doxorubicin is more likely due to AKBA-induced cellular changes rather than a physiochemical interaction between the two compounds. The control set of cells were allowed to grow without doxorubicin for 24 h while the others were being treated with AKBA. Doxorubicin was then added to both sets of cells. The EC_{50} of doxorubicin was remarkably lowered after pre-exposing cells to AKBA for 24 h where it was reduced from 104 ± 6 µM in the cells treated with doxorubicin alone to 0.7 ± 1 µM in the cells pre-exposed to AKBA prior to adding doxorubicin alone (Figure 2.9).

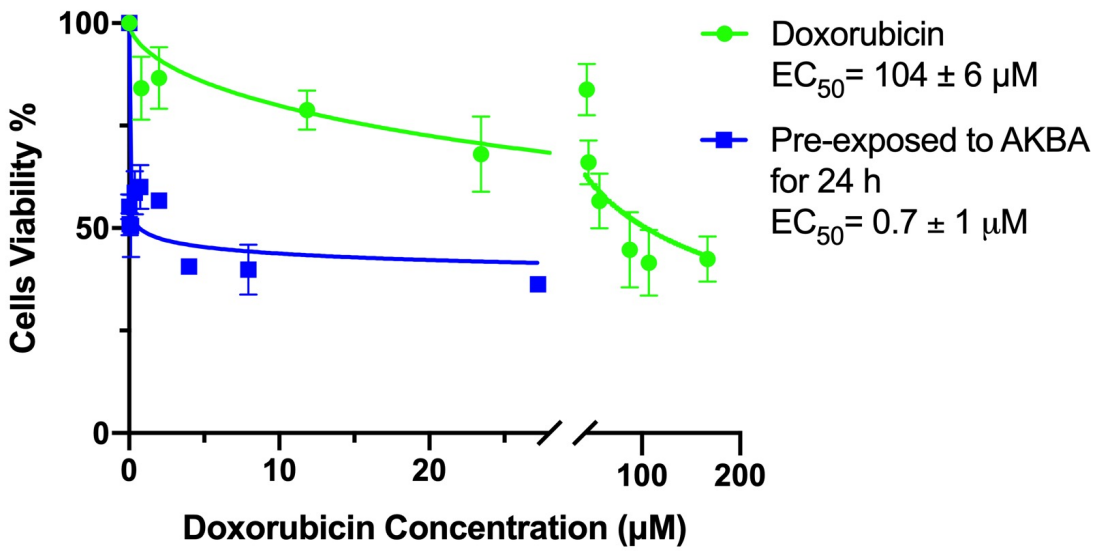


Figure 2.9: The effect of the pre-exposure to AKBA on doxorubicin toxicity on A2780ADR. *Data is shown as 3 independent experiments ±SEM.*

2.3.4 Discussion

Ovarian cancer is one of the leading causes of death among women cancers in the west (Jayson *et al.*, 2014). It is a difficult disease to treat for the reasons mentioned in the introduction such as the heterogeneity and the insufficiency of adequate diagnostic approaches in the early stages of the disease. The main approach to treat ovarian cancer involves chemotherapeutic agent regimens in addition to surgery. Chemotherapies cause a number of unpleasant and in some cases serious complications. Therefore, developing new agents to either treat, increase the efficacy of conventional chemotherapies at low doses or reduce the side effects of them is required. Plants were one of the natural sources of therapeutic agents thorough the history of developing medicinal agents. One of the plants used in traditional medicine against various diseases is frankincense (from *Boswellia sp.*). The possible effects of AKBA, a biologically active compound from frankincense, and extracts of frankincense using ethanol or water on ovarian cancer *in vitro* models and on conventional chemotherapies efficacy was explored in this project.

2.3.4.1 AKBA and frankincense extracts toxicity on ovarian cancer models

The ability of AKBA to effectively reduce the viability of ovarian cancer cells *in vitro* is shown using various cell lines, A2780, A2780cis, A2780ADR and OVCAR4. This confirms the results of previously unpublished work by K. Al Salmani *et al.*, who studied the effects of AKBA on ovarian cancer *in vitro,* for the first time using a number of ovarian cancer cell lines. In addition, the results in the current study has established the cytotoxicity of the 70 % ethanol extract of the resin towards A2780 and its resistant counterpart A2780cis (Figure 2.2 and 2.3). This could be a result of the presence of AKBA in the extract as shown using TLC (see appendix), which needs further confirmation and quantification using HPLC, for example.

In contrast, the water extract, to mimic one of the traditional ways in the preparation of the resin to treat diseases, was not toxic towards A2780 cell line. This could be investigated further by changing or adapting the extraction method. For example, frankincense essential oils extracted by hydrodistillation of *Boswellia sacra* gum resins was reported to be toxic towards breast and pancreatic cancers (Suhail *et al.*, 2011; Ni *et al.*, 2012).

2.3.4.2 Potential antagonism of cisplatin toxicity by AKBA

Potential antagonism between AKBA and cisplatin was observed in the isobologram results and confirmed statistically on a number of doses. This might occur due to physiochemical interaction between the compounds when they are present simultaneously resulting in loss of activity of cisplatin. It is important to note that the reduction in the toxicity of cisplatin due to the presence of AKBA occurs at doses higher than the EC_{50} of cisplatin on both A2780 and A2780cis but not on OVCAR4. The other possible reason for the reported antagonism is cytoprotection of cells that could result from induced Nrf2 induction. AKBA neuroprotection was attributed to the increased expression of Nrf2 (Ding *et al.*, 2014). Nrf2 over-expression has been reported to be a contributor to resistance of cells to cisplatin (Wang *et al.*, 2008) and its inhibition sensitised resistant ovarian cells to cisplatin (Cho *et al.*, 2008; Ji *et al.*, 2013). The role of Nrf2 in this phenomenon will be examined in the later chapters.

2.3.4.3 Improving the toxicity of doxorubicin by AKBA

Taking into account the CI values, synergistic (on A2780, A2780cis, OVCAR4 and pre-exposed A2780ADR) and additive (on A2780ADR) effects were observed after treating cells with a combination of AKBA and doxorubicin for 24 h. The toxicity of doxorubicin towards all tested cell lines was remarkably increased as a result of the presence of AKBA (26 μM). Moreover, AKBA sensitised the doxorubicin resistant cell line, A2780ADR, to doxorubicin. Khan *et al.* (2014) stated a synergistic effect of BSE in combination with doxorubicin against hepatocellular carcinoma. The synergistic interaction between AKBA and doxorubicin could be attributed to molecular changes following the exposure of the cells to the agents together. Cells were pre-exposed to AKBA for 24 h before adding a fresh medium and doxorubicin in an attempt to understand if the interaction is taking place on a molecular level or is a physiochemical reaction between the agents. The data (Figure 2.9) imply that the interaction is on a molecular level, since the EC_{50} of doxorubicin on A2780ADR was 100 fold less than the cells that were treated with doxorubicin but not pre-exposed to AKBA, whereas the difference between the EC_{50s} when the agents were added simultaneously compared to doxorubicin alone was approximately 25 fold. The synergistic interaction between AKBA and doxorubicin is a promising indicator that AKBA could be used as an adjuvant and doxorubicin doses could be reduced which then may lead to less side effect of the agent.

Other points worth highlighting are the toxicity of the dose of AKBA (26 µM) and the doubled EC_{50} value of A2780ADR in pre-exposure experiments. The cell viability of cell lines treated with 26 µM AKBA lies between 60 and 70 %. This may suggest that the improvement is due to the additive cytotoxicity of AKBA and doxorubicin together, not the synergistic interaction between the agents, which is at least of benefit. However, the CI values indicate a synergistic interaction between AKBA and doxorubicin. Moreover, there was antagonistic interaction between AKBA (26 µM) and cisplatin despite the toxicity of AKBA. In addition, the increased EC_{50} value of doxorubicin on A2780ADR could be attributed to background noise. The other proposed reason is the time that was given to cells to grow in the plate before exposing them to doxorubicin, which is 24 h more as described in section 2.2.4.

A number of molecular targets and cellular mechanisms could be involved in the synergistic interaction between AKBA and doxorubicin. For instance, both AKBA and doxorubicin were reported to induce cell cycle arrest at G1 and G2/M phase, respectively, as one of the mechanisms to trigger cell death in cancer cells (Liu, Huang and Hooi, 2006; Kim, Lee and Kim, 2009). Exploring such processes and targets could help reveal the molecular mechanisms behind the synergistic interaction. The effects of AKBA and doxorubicin on cell cycle, the mitochondria, NFκB pathway and P-gp protein expression will be investigated in the following chapters.

2.3.4.4 Paclitaxel and AKBA between additive and antagonistic interactions

Despite the increased toxicity of paclitaxel due to the presence of AKBA, the CI values indicate an additive effect on A2780 and slight antagonistic effect on A2780cis. This could be a consequence of the poor aqueous solubility of paclitaxel and lipophilic nature of both compounds (Rentea, 2008; Surapaneni, Das and Das, 2012). The exerted interaction of paclitaxel with AKBA resulted in the exclusion of it from further studies in this project. It lies between the synergistic interaction of doxorubicin and the antagonistic interaction of cisplatin with AKBA.

Chapter 3

Cell Cycle and Apoptosis

3 Cell cycle and apoptosis

3.1 Introduction

3.1.1 The cell cycle in normal and cancer cells

Eukaryotic cells replicate by a process called the cell cycle, which consists of phases recognised as G1, S, G2 and M (Figure 3.1). When cell division is required, cells inter the cell cycle at G1 from G0, a resting (quiescent) phase where cells are not dividing, or continue in the cycle from M phase. The G1 and G2 are called gap phases, where a number of checks are conducted before entering either the S or M phases, respectively. The S, synthesis, phase is when DNA replication occurs and the M, mitosis, phase is when cells divide. Cell cycle progression is strictly controlled by multiple checkpoints to ensure the readiness of a cell to enter the cell cycle or move from one phase to another (Deraitus and Freeman, 2001). For example, DNA replication will be delayed or stopped if DNA damage is detected in the S or M phase, then DNA repair will take a place or the cell will be forced to go through apoptosis. The control system is mainly driven by cyclin-dependent kinases (CDKs) and their inhibitors. The CDKs regulatory subunit are cyclins as the name suggests. Active CDK-cyclin complexes in the cell cycle control the transition between the phases which is finetuned by regulatory phosphorylation or dephosphorylation (Collins, Jacks and Pavletich, 1997).

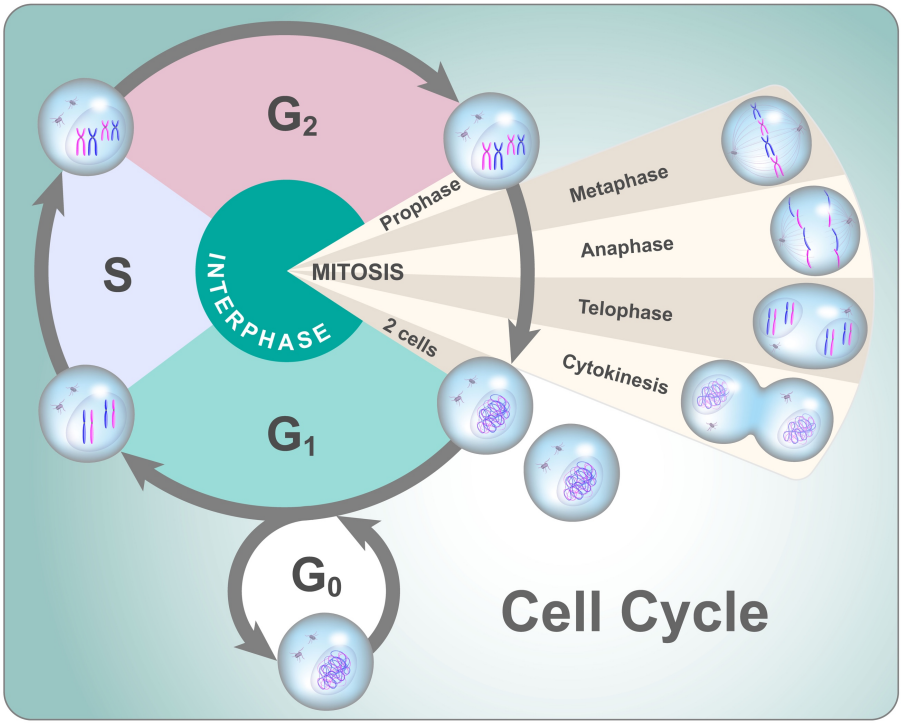


***Figure 3.1: Cell cycle phases**. (Shutterstock ID: 1251867079)*

Uncontrolled cellular proliferation and the lack of efficient checkpoints are parts of the hallmarks of cancer (Hanahan and Weinberg, 2000). Cell proliferation in cancer appears to be caused by the accumulation of genetic mutations that could induce uncontrolled cell growth, inhibit cell death and inactivate repair mechanisms (Vogelstein and Kinzler, 2004). Alteration in the cell cycle regulatory genes were sporadic across various types of ovarian cancer (D'Andrilli *et al.*, 2004). The reported alterations involve overexpression of cyclins (A, B, D1, D2 and E), CdKs (2 and 4), Cdcs (2, 25A and 25B) and p21, and down-regulation of p16, Rb, Rb2/p130, p27 and p57, and mutation in p53, and homozygous deletion of p15 (D'Andrilli *et al.*, 2004). This variability could be related to the heterogeneity of the ovarian cancer and the complexity of the cell cycle mechanism.

The therapeutic approaches to cancer, including ovarian, are developing towards more targeted therapies that target a specific gene, protein, pathway or mechanism. It is then rational to develop or investigate agents that target the cell cycle considering its crucial contribution to tumorigenesis. For example, aurora kinase inhibitors were reported to be effective against ovarian cancer *in vitro* and *in vivo* and, interestingly, an agent called MLN8054 is being examined in 'the management of patients with platinum-refractory or resistant epithelial, fallopian, or primary peritoneal carcinoma' (Campos and Ghosh, 2010).

3.1.2 Cell death in normal and cancer cells

In cell biology, there is more than one form of cell death, where variable mechanisms are involved. Controlled cell death is important in both physiological and pathological conditions. The established cell death types include, but are not limited to, apoptosis, necrosis, pyroptosis and autophagy, and the most recent discovered type is necroptosis (Figure 3.2) (Fink and Cookson, 2005; Dasgupta *et al.*, 2017). This brief introductory section will focus on apoptosis and may include necrosis, as necessary, in both normal and cancer cells, since this is the cell death process of primary consideration in this work.

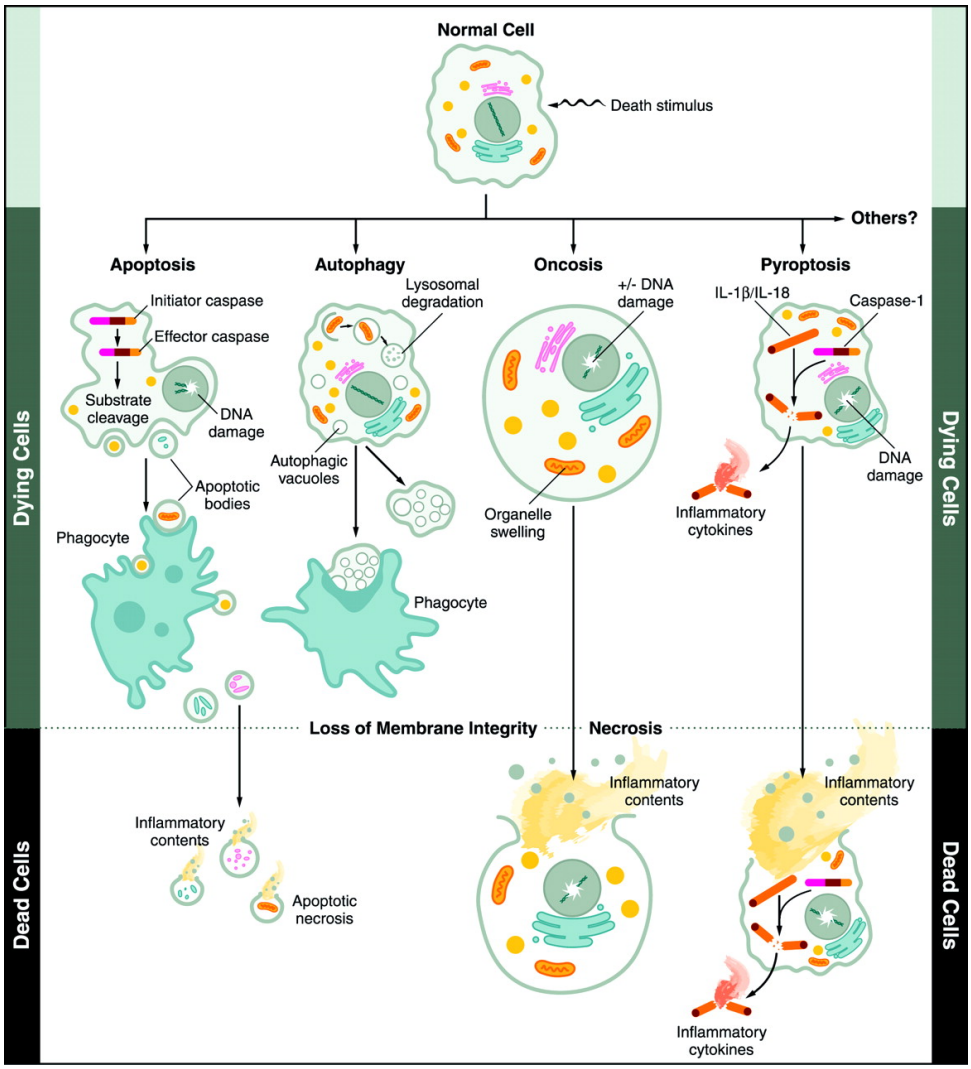


Figure 3.2: Cell death pathways in normal cells. *Taken from (Fink and Cookson, 2005).*

Apoptosis, the programmed cell death, is the most studied mechanism of cell death. It is a tightly controlled process that can occur during both physiological and pathological processes, which is the main feature that distinguishes apoptosis from necrosis, despite the recent indications that necrosis might also be a programmed cell death that occurs as a result of trauma or injury (Danial and Korsmeyer, 2004; Dasgupta *et al.*, 2017). Apoptosis is one of the ways to maintain homeostasis within tissues during embryogenesis, aging and healing, for example. Cell shrinkage and nuclear condensation are among the characteristics that differentiate apoptosis from necrosis. The key players in apoptosis are caspases, proteolytic enzymes. Caspase-2, -8, -9 and -10 are known as the initiator caspases, which generally activate the executioner caspases that include caspase-3, -6 and -7 (Dasgupta *et al.*, 2017).

The apoptosis pathway can be divided into extrinsic and intrinsic pathway (Figure 3.3). The extrinsic pathway occurs as a response to extracellular stimuli of death receptor, which is also referred to as death receptor pathway. Cell death in this pathway happens as a result of the catalytic activity of caspases-8 and/or -10, which could be induced following a series of upstream multi-protein death inducing signalling. This starts from triggering cell surface death receptors by the binding to specific members of tumour necrosis factor (TNF) and, similar to the other pathways, ends in the catalytic activation of the executioner caspase-3 (Ly, Grubb and Lawen, 2003; Elmore, 2007; Hotchkiss *et al.*, 2009). The other examples of cell surface death receptors that can activate the extrinsic pathway include FasR and TRAIL.

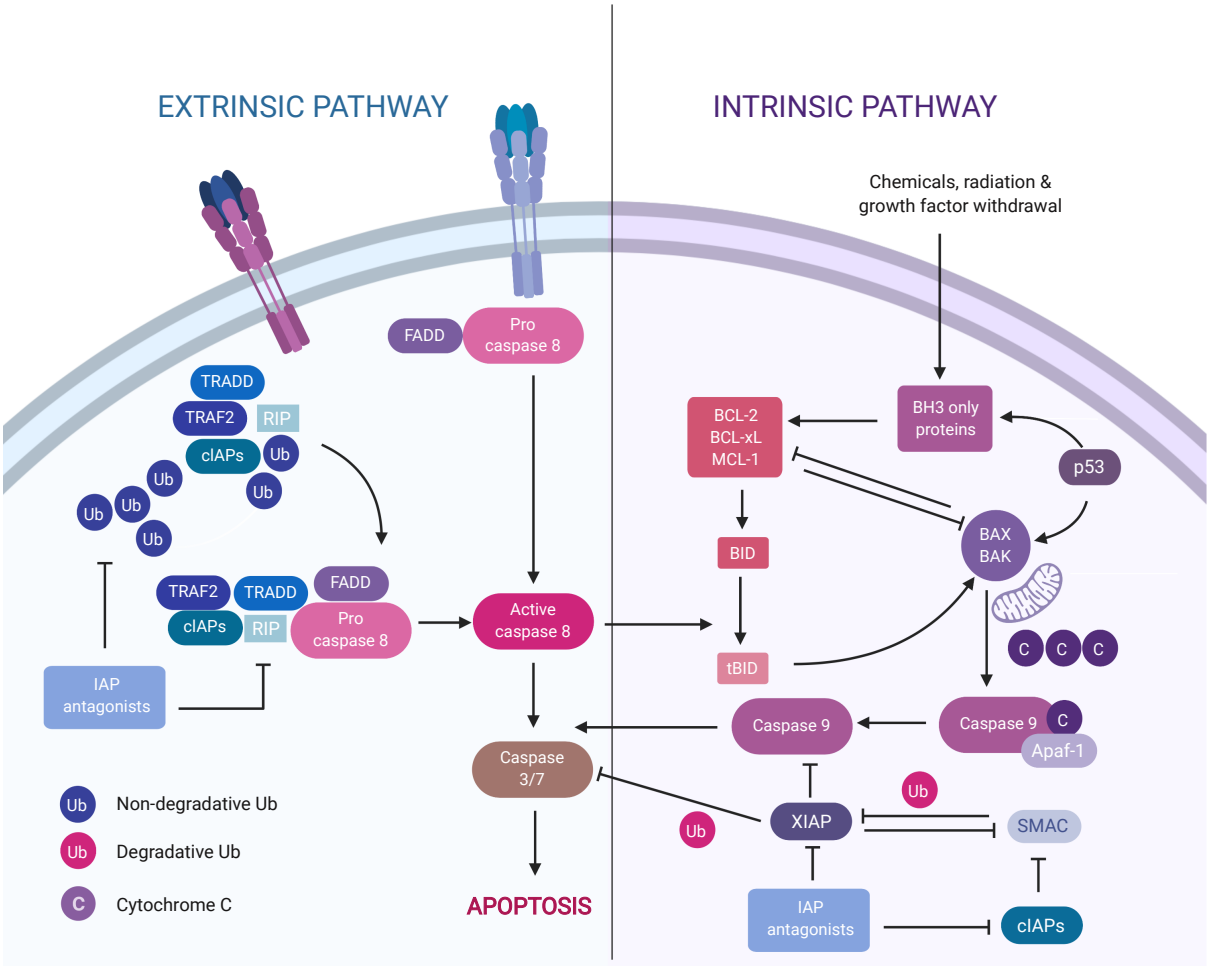


Figure 3.3: Extrinsic and intrinsic apoptosis. *Created using Biorender.*

On the other hand, the intrinsic pathway, also known as mitochondrial pathway, is regulated by both pro- and anti-apoptotic members of the Bcl-2 protein family. This pathway is normally activated in a response to intracellular signals of cell damage such as increased levels of reactive oxygen species or DNA damage. The caspase playing a key role in the initiation of the intrinsic pathway in a response to the intracellular signals of the cell damage is caspase-9. Caspase-9 can also be triggered by cytochrome C released from disrupted mitochondria, which could initiate or result from apoptosis. Therefore, mitochondrial membrane permeability has been used as an indicator of the early stages of apoptosis or apoptosis in general (Ly, Grubb and Lawen, 2003; Dasgupta *et al.*, 2017). There is 'cross-talk' between the intrinsic and extrinsic apoptotic pathways, with caspases associated with one pathway activating caspases in the other.

The importance of understanding apoptosis in cancer relies on the fact that evading apoptosis is one of the hallmarks of cancer mentioned by Hanahan and Weinberg (2000). As dicussed

above, cell cycle arrest or the induction of apoptosis are the expected consequence of errors in cell cycle in healthy cells, which is impaired in most cases of tumourgenisis. Overexpression of pro-survival Bcl-2 was reported in about 50 % of human cancers, which may rseulted from oncogenes that dyregulate expression of this protein (Dasgupta *et al.*, 2017). The other example of the molecular causes of the defect of apoptosis in cancer is mutations in TP53, which intiate apoptosis in response to DNA damage, in a number of neoplasms including HGSOC (Hotchkiss *et al.*, 2009). The induction of apoptosis in cancer cells is one of the main effects exploited in cancer chemotherapy.

3.2 Methods

3.2.1 Cell cycle analysis

The effect of AKBA alone and combined with either cisplatin or doxorubicin on cell cycle phases was assessed on a flow cytometer by quantifying DNA staining. DNA contents were stained with propidium iodide (PI) after fixing cells with 70 % v/v cold ethanol. PI is a red-florescent dye that can stain both DNA and RNA in either dead or permeabilised cells. Since the experiment is based on analysing DNA content, ribonuclease was used to remove RNA from tested samples. Four phases of cell cycle G1, S (DNA synthesis phase), G2 and M (mitosis phase) can be detected using this approach based on the brightness of the signal. The brighter the signal is the higher the DNA contents are.

Cells were counted and seeded at the optimal cell density (5 x 10^5 cells per well in a 12-well plate) and allowed to attach for 24 h at 37 °C in 5 % CO_2. The medium was replaced with fresh medium and various concentrations of AKBA, doxorubicin, cisplatin, AKBA (26 µM) combined with doxorubicin or AKBA (26 µM) combined with cisplatin were added. Two controls were included which are negative (untreated) and positive (etoposide). Cells were exposed to the tested agents for 24 h since the toxicity experiments were performed, mainly, at this time point. Moreover, the reported doubling time of A2780 cells is about 18 to 22 h (Beaufort *et al.*, 2014). After 24 h, the medium was taken into fresh tube and the cells were trypsinised and combined with the supernatant from the same well. Cell suspensions were centrifuged at 600 x g for 5 minutes. The supernatant was removed and the cells were suspended in cold PBS as a wash, this was repeated once, cell were pelleted at 600 x g for 5 minutes each time. Then, 70 % v/v ethanol in dH_2O was used to fix the cells, by adding dropwise to the cells with gentle vortexing to allow single cell suspension formation. Fixed cells were then stored at -20 °C until PI staining and flow cytometry. For staining, fixed cells were washed twice with PBS, cells pelleted at 600 x g for 5 minutes. 500 µL of PBS was slowly added to the washed cells with gentle tapping. Then cells were treated with ribonuclease A (RNase A) (2.5 µL of 10 mg/mL) to ensure that DNA, not RNA, is stained. To stain the cells, 25 µL of 1 mg/mL PI was added to each tube. The tubes were incubated at 37 °C for 30 minutes in the dark. Then, the tubes were placed on ice immediately and analysed by flow cytometry. Stained DNA was analysed/quantified using a BD Accuri C6 Plus flow cytometer (BD Biosciences, USA). The resulted data was exported and then

analysed using FlowJo (V10, USA) (Figure 3.4). A total of 30,000 of events were acquired for each sample. Results were obtained in duplicate from three independent experiments.

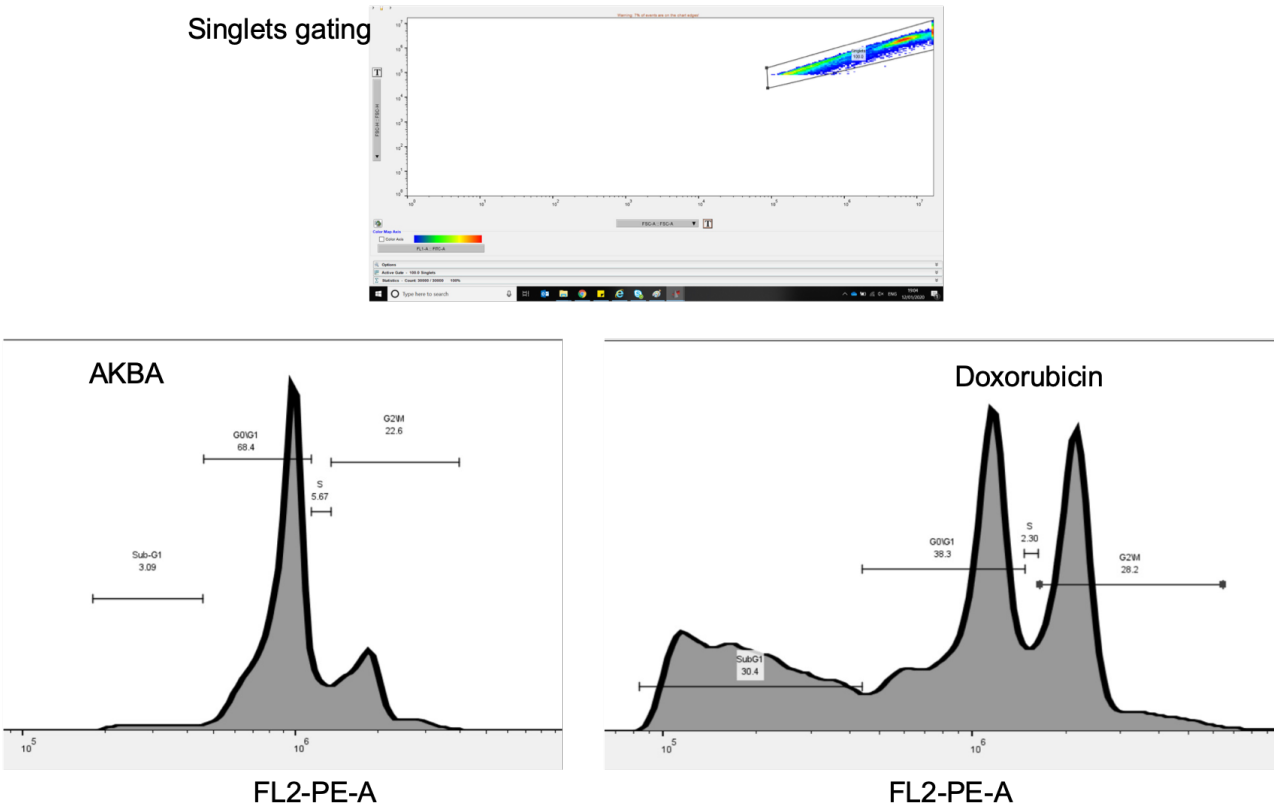


Figure 3.4: Example flow cytometry histogram showing different phases of the cell cycle.

3.2.2 Polarisation of the mitochondrial membrane

JC-10 Mitochondrial Membrane Potential (MMP, $\Delta\psi_m$) Assay Kit (ab11213, Abcam) was used to assess the effect of AKBA, cisplatin, doxorubicin and AKBA combined with either of the chemotherapies on mitochondria. JC-10 is a fluorescent cationic and lipophilic dye which aggregates in mitochondria if the mitochondria have an intact proton gradient (red fluorescence). In apoptotic and necrotic cells, the dye will diffuse out of mitochondria, forming green fluorescent staining.

Cells were counted and seeded at 4 x 10^4 cells per well in a black 96-well microplate with clear bottom. Cells were allowed to attach and acclimatise for 24 h, 37 °C , 5 % CO_2. The old medium was then removed and replaced with fresh phenol red free RPMI-1640 medium + 10 % NCS. Cells were exposed to various concentrations of AKBA, doxorubicin, cisplatin or single dose of AKBA (26 μM) combined with either doxorubicin or cisplatin for 24h. To stain cells, the medium was replaced with PBS and the recommended volume of JC-10 containing assay solution was added (see Appendix $\Delta\psi_m$ assay for protocol). The plate was incubated for 45 mins at 37 °C in 5 % CO_2 before adding 50 μL of the assay buffer B. Finally, fluorescence intensity was monitored at excitation/emission (475/500-550 and 520/580-640 nm) using a microtitre plate reader (GloMax® Discover System, GM3000, Promega Corporation, USA). These were the nearest values to the recommended ones: exitation/emission (490/525 and 540/590 nm). The ratio of 490/540 of each sample was calculated and then normalised to percentage against the negative control (untreated).

3.2.3 Statistical analysis

The statistical significance was tested using ordinary one-way ANOVA (Tukey multiple comparison statistical test) on GraphPad Prism 8. If $P<0.05$, the result was considered statistically significant. To compare the difference between single doses in the presence or absence of AKBA, unpaired t-tests using GraphPad Prism (version 7.0b for Mac OS X, GraphPad Software, La Jolla California USA, www.graphpad.com) was used. $P<0.05$ was considered significant.

3.3 Results

3.3.1 Cell cycle

3.3.1.1 The effect of AKBA on the cell cycle on A2780

After treating cells for 24 h with AKBA, cell cycle arrest at G0/G1 was observed at the highest concentration of AKBA which was 62 µM (Figure 3.5). The difference to the control G0/G1 was statistically significant ($P<0.0001$). Moreover, the population at sub-G1 was considerably higher at the same concentration of AKBA (34 ± 9 % of the events) compared to untreated cells (4 ± 1 % of the events) and etoposide (5 µM, 13 ± 1 % of the events), topoisomerase inhibitor chemical, which was used as a positive control for the reported activity of the induction of cell cycle arrest at G2/M. This could be used as an indicator of the cell death caused by AKBA as it is a representative of the fragmented DNA (Table 3.1). The distribution of the cell cycle following the exposure of the cells to the other doses of AKBA was similar to the untreated cells.

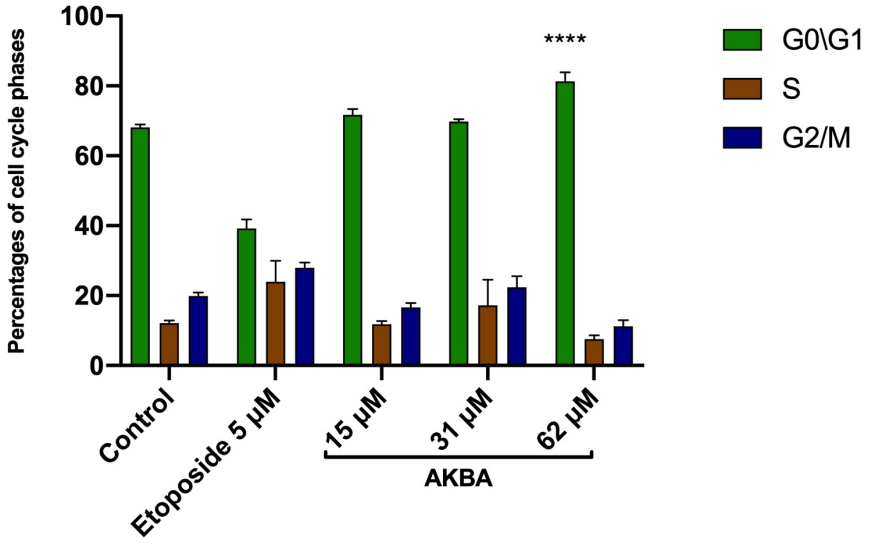


Figure 3.5: Cell cycle distribution of A2780 exposed to AKBA for 24 h. *The mean of 3 independent experiments ± SEM is shown. **** is P<0.0001 compared to control.*

3.3.1.2 The effects of cisplatin alone or combined with AKBA on the cell cycle of A2780 cells

Cells were also exposed to various concentrations of cisplatin for 24 h. Cell cycle was arrested at S phase at all tested concentrations 12, 15 and 23 µM (Figure 3.6). The difference to the control was statistically significant at both 12 and 23 µM doses. The percentage of the fragmented DNA at sub-G1 was elevated following treating A2780 cells with cisplatin for 24 h. The percentages were 11, 29 and 38 % for 12, 15 and 23 µM, respectively (Table 3.1).

In addition, cells were treated with 26 µM AKBA which was added simultaneously with cisplatin. Interestingly, the effect of cisplatin on S phase at 12 µM was significantly decreased and the cell cycle distribution was not significantly different to the control. The effect of the presence of AKBA with the other two doses was not statistically significant. Finally, the percentages of the events at sub-G1 of samples treated with cisplatin and AKBA were less than the ones treated with cisplatin alone at both doses 15 and 23 µM, which were 18 and 13 % (Table 3.1).

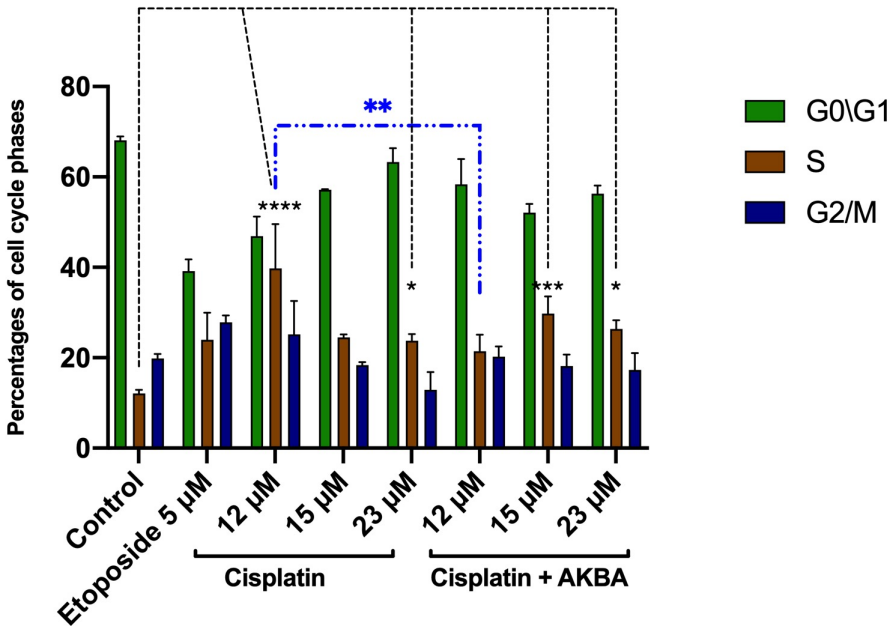


Figure 3.6: Cell cycle distribution of A2780 exposed to cisplatin alone or combined with AKBA for 24 h. *The mean of 3 independent experiments ± SEM is shown. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 compared to control. The significant difference between the presence and absence of AKBA at 12 µM is shown in blue.*

3.3.1.3 The effects of doxorubicin alone or combined with AKBA on the cell cycle on A2780

The effect of doxorubicin on the cell cycle was assessed by exposing A2780 cells to doxorubicin or doxorubicin combined with AKBA for 24 h. Cell cycle arrest was reported at G2/M phase, which was statistically different to the control (Figure 3.7). Moreover, the percentages of the events at sub-G1, *i.e.* DNA fragments, were increased following the exposure to doxorubicin (Table 3.1). The presence of AKBA did not affect the changes to the cell cycle that caused by doxorubicin neither the percentages of the events at sub-G1. The difference between the presence or absence of AKBA was not statistically significant at all tested doses.

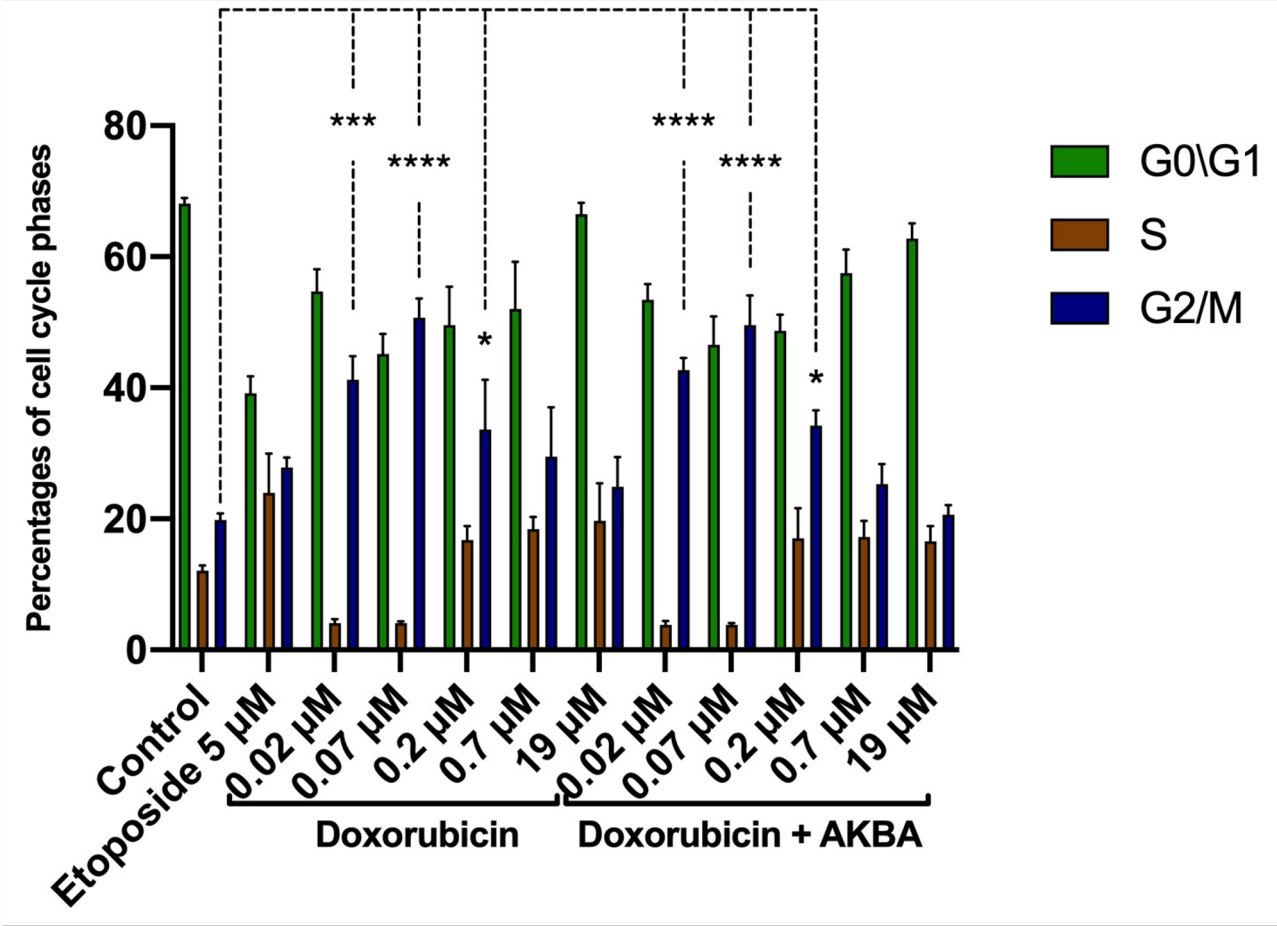


Figure 3.7: Cell cycle distribution of A2780 exposed to doxorubicin alone or in combination with 26 uM AKBA for 24 h. *The mean of 3 independent experiments ± SEM is shown. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 compared to control.*

Table 3.1. The percentages of sub-G1 of A2780 cells exposed to various agents for 24h.

Sample	Dose (μM)	Sub-G1 %	SEM
Control	-	4	1
Etoposide	5	13	1
AKBA	15	3	2
	31	1	0
	62	34	9
Cisplatin	12	11	3
	15	29	6
	23	38	14
Cisplatin + 26 μM AKBA	12	14	5
	15	18	8
	23	13	2
Doxorubicin	0.02	10	2
	0.07	16	5
	0.2	26	6
	0.7	30	7
	19	7	2
Doxorubicin + 26 μM AKBA	0.02	12	3
	0.07	14	4
	0.2	17	5
	0.7	24	4
	19	16	10

3.3.2 Mitochondrial Membrane Potential (MMP, $\Delta\psi_m$)

3.3.2.1 Single agent exposures and mitochondria depolarisation

The effects of various treatment approaches on $\Delta\psi_m$ was assessed using JC-10 (fluorescent lipophilic dye). AKBA has significantly changed the $\Delta\psi_m$ at the highest concentrations (61 and 72 µM) after exposing A2780 cells for 24 h. Mitochondrial depolarisation was also reported on the cells treated with cisplatin alone at 19 and 28 µM, which was significantly different to the untreated control. On the other hand, there was no significant change in the $\Delta\psi_m$ following the exposure to a range of doxorubicin doses for 24 h (Figure 3.8).

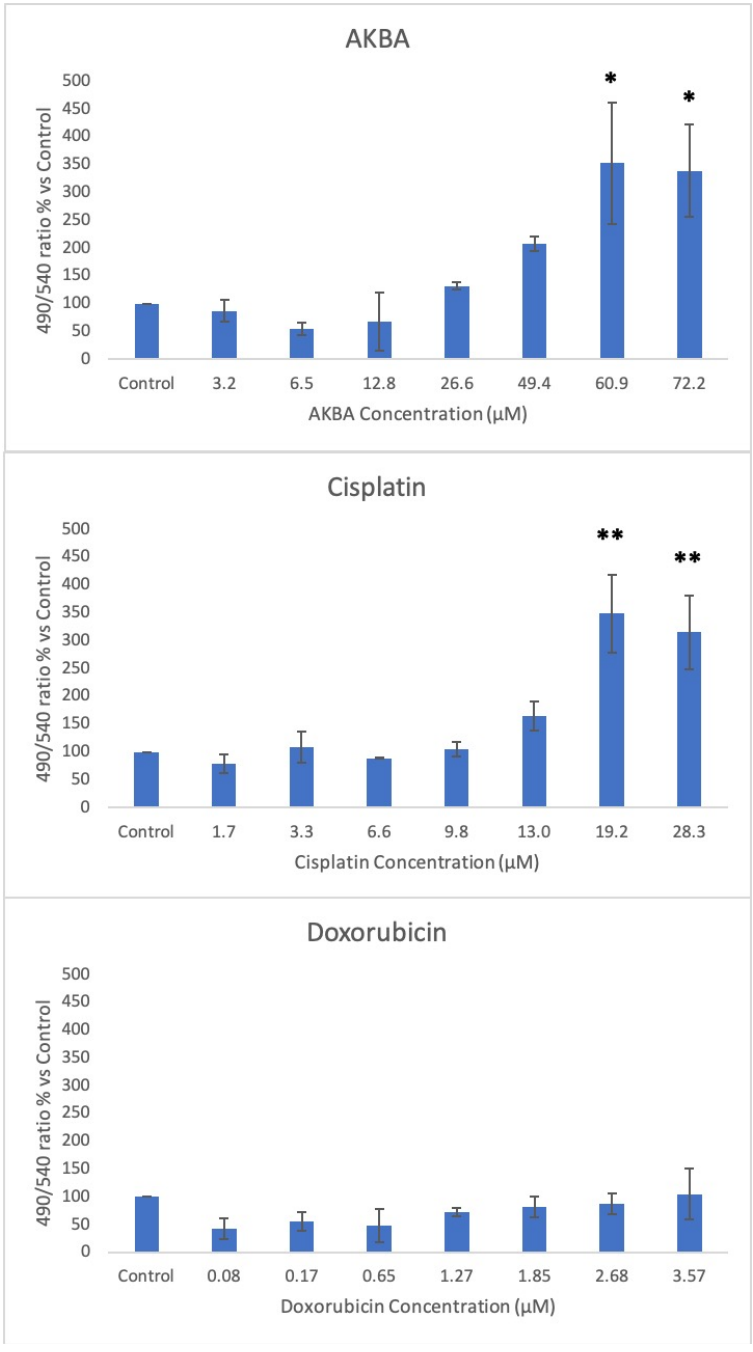


Figure 3.8: The effects of AKBA, cisplatin and doxorubicin on mitochondrial membrane potential on A2780. *Cells were exposed to different concentrations of each tested compounds for 24 h. Data shown as the mean of n=3 independent experiments ± SEM. *P<0.05 and **P<0.01 compared to control.*

3.3.2.2 Cisplatin or doxorubicin combined with AKBA on $\Delta\psi_m$

In an attempt to investigate the effect of combining AKBA with either cisplatin or doxorubicin on the changes to the $\Delta\psi_m$ that was caused by exposing A2780 cell to one of the agents alone, cells were treated with 26 µM AKBA combined with various concentrations of cisplatin or doxorubicin for 24 h. The impact of the presence of AKBA with cisplatin on the effect of the high concentrations of cisplatin on the $\Delta\psi_m$ was negligible and was not statistically different compared to the same doses without AKBA. On the other hand, there was a significant increase in the mitochondrial membrane depolarisation as a result of combining AKBA with doxorubicin compared to doxorubicin alone (Figure 3.9).

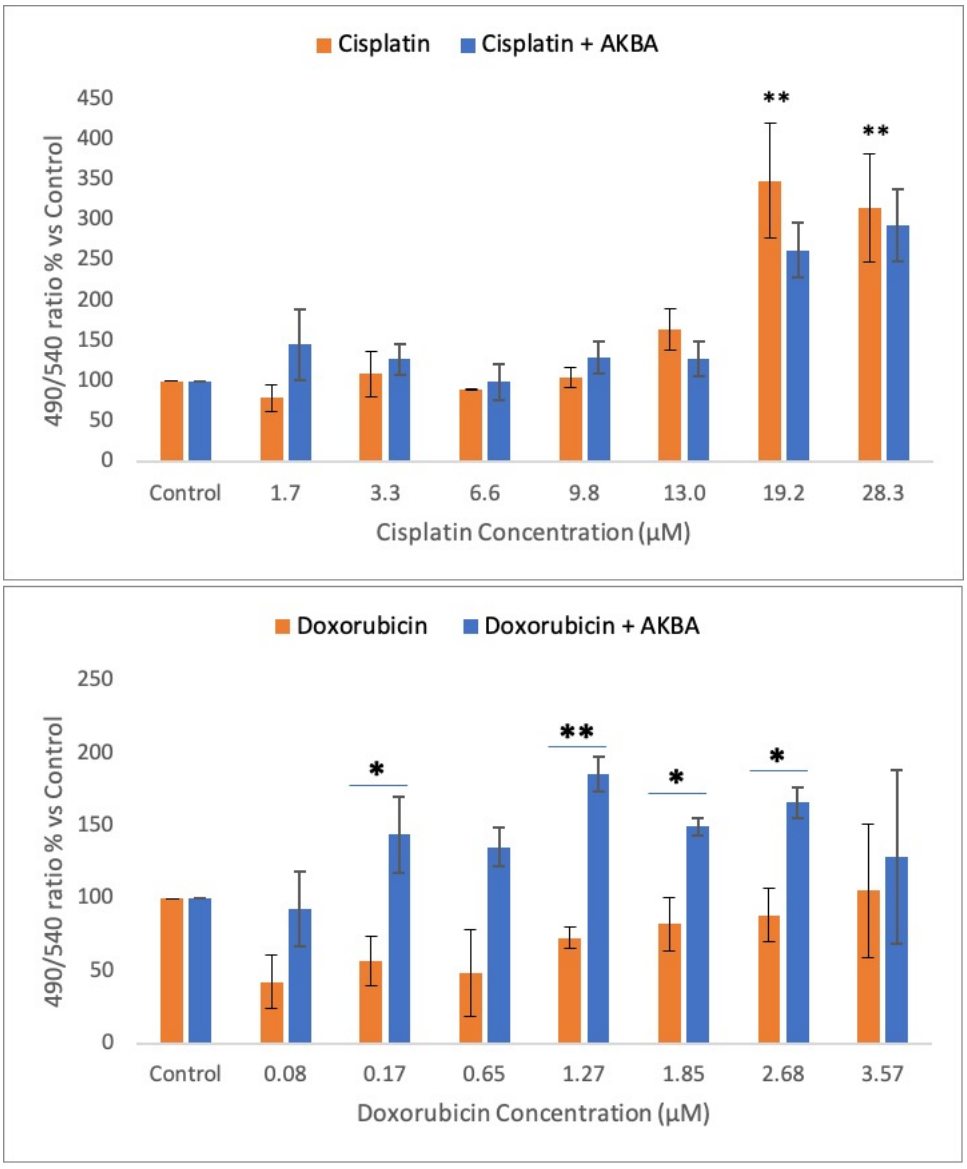


Figure 3.9: The effects of AKBA combination on the effects of cisplatin or doxorubicin on mitochondrial membrane potential on A2780. *Cells were exposed to different concentrations of each tested compounds for 24 h combined with 26.55 µM AKBA. Data shown as the mean of n=3 independent experiments ± SEM. **P<0.01 compared to the control for the cells exposed to cisplatin. *P<0.05 and **P<0.01 compared to the same dose in the absence of AKBA for the cells exposed to doxorubicin.*

3.4 Discussion

3.4.1 The effect of AKBA, cisplatin and doxorubicin alone and in combination on cell cycle

The cell cycle consists of four phases, G0/G1, S, G2 and M, and it is normally tightly regulated by a number of signalling pathways, checkpoints, kinases and other proteins. The impaired control of the cell cycle is one of key features of cancer. Targeting the cell cycle in cancer is one of the therapeutic approaches to either decelerate tumour growth or to induce cell death, either apoptosis or necrosis. The effects of tested agents, AKBA, cisplatin and doxorubicin, on the cell cycle were examined in this chapter.

Cell cycle arrest at G1 was shown as a result of exposing A2780 cells to AKBA for 24 h. Liu, Huang and Hooi (2006) show that AKBA induced cell cycle arrest at G1 in colon cancer models, which was linked to the downregulation of G1 cyclins and CDKs by AKBA through p21 dependent pathway. The ability of AKBA to inhibit cellular proliferation could be, in part, a result of the induction of cell cycle arrest. The other noticeable effect is the amount of the fragmented DNA (sub-G1), which was considerably higher for the sample treated with AKBA compared to the control. This is reflective of cell death and another contributor to the inhibition of cellular growth (Chashoo *et al.*, 2010; Lüpertz *et al.*, 2010).

Cisplatin induced cell cycle arrest at S phase, the DNA synthesis phase, with a reduction in the percentages of cells at G1 and G2/M phases. Cell cycle arrest at S phase after exposing HL-60, a human leukemic cell line, to low doses of cisplatin was reported by Velma, Dasari and Tchounwou (2016). They also show an accumulation of the cells at sub-G1 in a time dependent manner. In the current project, the increase of the signal at sub-G1 was dependent on the dose of cisplatin.

The concurrent presence of AKBA with cisplatin interfered with the effect of cisplatin on cell cycle. The accumulated cells at S phase that was shown at 12 μM cisplatin was significantly decreased due to the presence of AKBA. AKBA also resulted in the reduction of fragmented DNA at sub-G1 when it was added with 15 and 23 μM cisplatin. These observations concur with the previous antagonistic interaction that was shown in the previous chapter (see 2.2.2.1).

The distribution of the cell cycle was significantly changed after exposing A2780 cells to doxorubicin for 24 h, where the cell cycle was arrested at G2/M phase. The percentages of the events at sub-G1 in the samples exposed to doxorubicin were higher than the control.

Lüpertz *et al.* (2010) state that doxorubicin induced cell cycle arrest at G2 (1 μM) and G0/G1 (5 μM) in a dose and time dependent manner on a human colon carcinoma cell line, Hct-116, with an increase in the amount of fragmented DNA at sub-G1 with the lower dose of doxorubicin. Adding AKBA with doxorubicin did not lead to any changes to the effect of doxorubicin on the cell cycle, neither on the accumulated DNA fragments at sub-G1.

3.4.2 The effect of AKBA, cisplatin and doxorubicin alone and in combination on $\Delta\psi_m$

Mitochondria are vital cellular organelles that are mainly responsible for ATP generation in eukaryotic cells. Mitochondria are key players in the intrinsic apoptotic pathway for the fact that the permeabilisation of its outer membrane leads to cytosolic release of proapoptotic proteins. The release of cytochrome c, for example, from the disrupted mitochondrial outer membrane to the cytoplasm can initiate apoptosis by inducing a conformational changes to apoptotic protease-activating factor-1 (APAF-1) protein which then bind to and activate caspase 9 to form a complex called apoptosome (cytochrome c, APAF-1 and caspase 9) (Dasgupta *et al.*, 2017).

Mitochondria have emerged as one of the targets for cancer treatments considering their role in cell survival and in apoptosis in both normal and pathological conditions. 'Mitochondrial control of apoptosis has been described at several levels: (1) maintenance of ATP production and (2) $\Delta\psi_m$ and mitochondrial membrane permeability for the release of certain apoptogenic factors from the intermembrane space into the cytosol' (Ly, Grubb and Lawen, 2003). In this chapter, the changes to the $\Delta\psi_m$ following the exposure to tested agents and the combination approaches were explored.

The impact of AKBA on the $\Delta\psi_m$ was significant at the high doses after exposing A2780 cells for 24 h. Changes in the $\Delta\psi_m$ was observed by Lu *et al.* (2008) after exposing PC-3, a human prostate cancer cell line, to AKBA. Qurishi *et al.* (2012) reported a loss of $\Delta\psi_m$ in HL-60 cells that resulted from the exposure to 3- α -propionyloxy-β-boswellic acid (POBA), a synthetic derivative of β-boswellic acid. The loss of $\Delta\psi_m$ could be an indicator of the induction of apoptosis by creating pores and releasing proapoptotic proteins, such as cytochrome c, in the cytoplasm.

Similar to AKBA, the $\Delta\psi_m$ was changed after exposing A2780 cells to cisplatin for 24 h. This observation complies with previously published data on the effect of cisplatin on the $\Delta\psi_m$ such as the one published by Xu *et al.* (2018) where changes in the $\Delta\psi_m$ was observed in

SKOV3 cells exposed to cisplatin. The depolarization of the mitochondria that was caused by cisplatin was not affected by the presence of AKBA. More repeats of the experiment, applying various experimental approaches and time points may lead to a more definitive conclusion.

Finally, there were negligible changes in the $\Delta\psi_m$ towards the hyperpolarisation of the mitochondrial membrane following the exposure of A2780 cells to doxorubicin for 24 h. An opposite effect, that is mitochondrial membrane depolarisation, was noticed on a number of cancer cell lines including SKOV3 exposed to doxorubicin (Rogalska *et al.*, 2011). This contradiction could be linked to the differences between cell lines or to the lack of data on other time points in this project. It is worth mentioning that hyperpolarisation is one of the known causes of the swelling of mitochondria that leads to their rupture and the release of proapoptotic proteins (Ly, Grubb and Lawen, 2003).

The presence of AKBA significantly changed the effect of doxorubicin on $\Delta\psi_m$ leading to mitochondrial membrane depolarisation despite the fact that the tested dose of AKBA itself did not shown any effect on $\Delta\psi_m$. This joint impact on mitochondria could be one of the molecular explanations of the synergistic interaction between AKBA and doxorubicin.

Chapter 4

Nuclear Factor kappa-light-chain-enhancer of activated B cells (NFκB) pathway

4 Nuclear Factor kappa-light-chain-enhancer of activated B cells (NFκB) pathway

4.1 Introduction

Alongside carcinogens, inflammation is linked to carcinogenesis with estimates that 'inflammation contributes to the development of about 15 % of all cancer' (Marx, 2004). The increase in liver cancer risk in patients with chronic hepatitis B infections is one of the classical examples that support this view (Marx, 2004). The review by Hanahan and Weinberg (2011) discusses the tumour promoting inflammation as one of the hallmarks of cancer. They stated a number of pieces of evidences from literature of the involvement of inflammation in promoting cancer such as providing tumours with growth factors to progress and survive and enzymes that enable tumours to metastasise.

In an attempt to find a possible molecular link between inflammation and cancer, researchers investigated DNA transcription factors of NFκB protein family (NFκB1/p105, NFκB2/p100, RelA/p65, RelB and c-Rel), which are widely known as a hallmark of inflammation (Figure 4.1). NFκB was found in its active form in a variety of cancers even though oncogenic mutations were limited to lymphoid malignancies. Apoptosis inhibition, cell proliferation stimulation and enhancing migratory and invasive phenotype were found to be the effects of the activated NFκB in tumour progression. It is not only NFκB that is involved in the link between inflammation and cancer, other members of NFκB pathway could play a role in either NF-κB-dependent or -independent manner (Karin, 2009). For example, the mTOR pathway can be activated by Tuberous sclerosis proteins 1 (TSC1) following phosphorylation by IKKβ, a regulatory kinase of NF-κB, which leads to enhanced angiogenesis and contributes to tumour development (Israël, 2010).

Ovarian cancer is no exception in terms of the contribution of the activated NFκB to its development. For instance, the impairment in the ability of tumour-associated macrophages to respond to M1 activation signals in human ovarian carcinoma was associated with activation of the NFκB p50 homodimer (Kaltschmidt *et al.*, 2019). P50, a cleaved product of P105, is inactive by itself. It needs to form a heterodimer with RelA, RelB or c-Rel or a P50/P50 homodimer to be active (Yu, Wan and Huang, 2009). In addition, the poor outcome and aggressiveness of ovarian cancer was found to be linked to the activation of NFκB

through IKKβ (Hernandez *et al.*, 2010). The NFκB pathway is a logical target for treating and preventing cancer considering its role and relative importance in cancer cell biology.

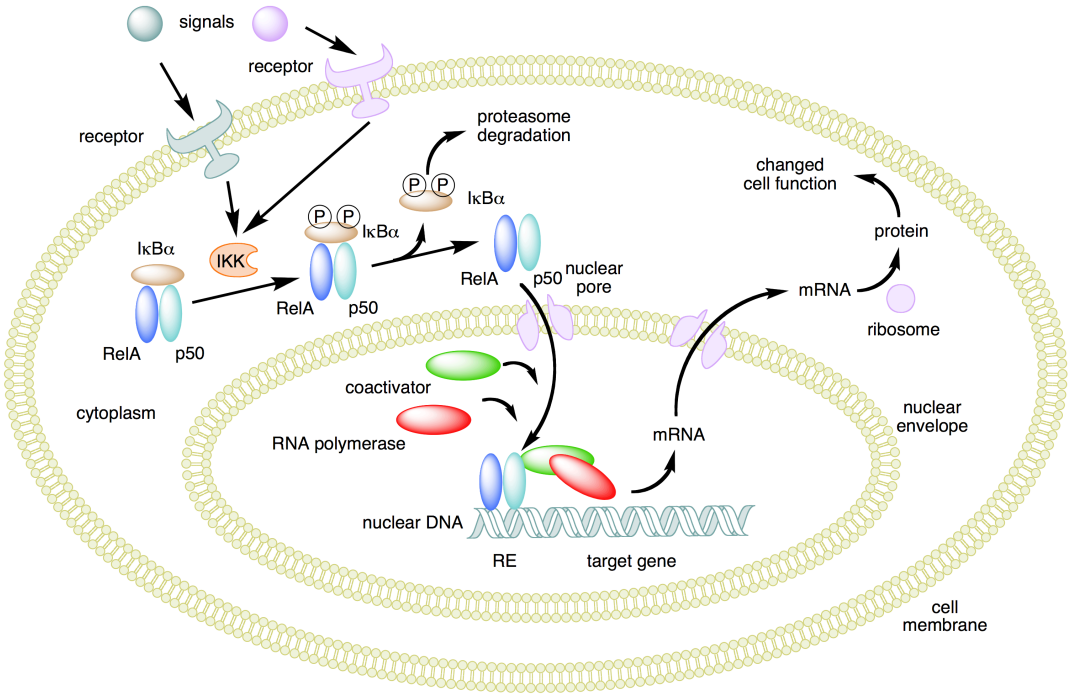


Figure 4.1: NFκB signalling pathway. *(https://commons.wikimedia.org)*

The synergistic interaction between AKBA and doxorubicin was established in the previous chapters. Therefore, the aim of this chapter is to investigate the effects of AKBA, doxorubicin and the combination of both agents on the NFκB pathway as one of the possible molecular targets of the combination of AKBA and a chemotherapy drug and possibly help in explaining the molecular action of AKBA.

4.2 Methods

4.2.1 Proteome Profiler Human NFκB Pathway Array

Proteome Profiler Human NFκB Pathway Array (Catalogue Number: ARY029, R&D systems, USA) was used to screen the effect of AKBA (26.55 μM), doxorubicin (70 nM) and both agents combined together on the expression of NFκB pathway protein. The array is provided with only four wells and its cost is relatively high. Therefore, a few samples were used without a positive control. The array facilitates the simultaneous detection of 41 human proteins and 4 serine or tyrosine phosphorylation sites. A duplicate of capture and control antibodies were spotted on nitrocellulose membranes.

The provided protocol was followed to test the effect of candidate agents on the NFκB pathway, see the appendix. The only difference to the original protocol is the number of cells seeded. The cell density per well was 3 x 10^5, which was based on western blot optimisations for A2780 (not shown). In brief, cell lysates were collected after exposing the seeded cells to the agents for 24 h, a timepoint which was chosen based on previous experiments to be a starting point. The lysates were then diluted and incubated overnight with the Human NFκB Pathway Array. The array was washed to remove unbound proteins and incubated with a cocktail of biotinylated detection antibodies. Streptavidin-HRP and chemiluminescent detection reagents were applied. The GeneGnome - Syngene bio imaging with GeneSys V1.4.1.0 software was used to take chemiluminescence images. The density of the spots were determined using densitometry via the ImageJ software. A reference co-ordinate provided by the supplier that indicates the position of the screened protein was followed to collect and analyse data.

4.2.2 Western Blotting

Western blotting is a technique that separates proteins based on molecular weight through the use of denaturing polyacrylamide gel electrophoresis (PAGE) to separate a mixture of proteins which are then transferred to a membrane. A protein of interest can be identified based on the molecular weight and use of an antibody targeting a selected protein along with a secondary antibody coupled to a reporter protein, such as horseradish peroxidase.

4.2.2.1 Equipment and materials

All materials were obtained from Thermo Fisher Scientific (Loughborough, UK), unless noted otherwise.

- Mini Gel tank and electrophoresis power supply (Invitrogen, A25977).

- Running Buffer.

- Bolt 4 - 12 % Bis-Tris Plus Gel.

- iBlot™ 2 Gel Transfer Device.

- iBlot™ 2 Nitrocellulose Regular Stacks.

- Roller, scissors and forceps.

- Paper towels.

- Containers for washing and staining the blots.

- Rocker.

- Gel imager.

- Acetate sheets.

- Cling film.

- Pipettes and tips.

- Glass bottles.

- Bovine Serum Albumin (BSA).

- Primary antibody.

- Secondary antibody.

- 6 well plates.

- Cell scrapers.

4.2.2.2 Solutions preparation

- 10 x Tris-buffered saline (TBS): 500 mM Tris base, 1.5 M sodium chloride. Adjust pH to 7.5 using HCl.

- 20% v/v Triton X-100 in dH2O.

- TBS-Tx100: 450 ml dH2O + 50 ml 10 x TBS + 2.5 ml 20% Triton X-100 (or 0.5 Triton X-100).

4.2.2.3 Sample preparation

A2780 cells were seeded in 6 well plates and allowed attach and acclimatise for 24 h at 37 ºC in 5 % CO_2. The seeding numbers were 6 x 10^5 cells per well for 1 h exposure and 3 x 10^5 cells per well for 24 h exposure. The old medium was removed and replaced with fresh medium. Test agents were then added to expose the cells for the proposed period of time, 1 h or 24 h. The concentrations of the agents used in this experiment were based on the MTT data. After exposing cells for the desired time, the medium was removed and 72 µL of lithium dodecyl sulfate (LDS) sample buffer was added to lyse the cells and denature proteins. Well contents were scraped using disposable scrapers and collected in 1.5 mL Eppendorf tubes. Each cell lysate was sonicated using a Qsonica sonicator for 15 seconds at 20% pulse amplification. The required volume (8 µL) of NuPAGETM Sample Reducing Agent was added to each sample just before adding the samples to the gels for electrophoresis.

4.2.2.4 Gel electrophoresis

Precast BoltTM 4-12% Bis-Tris Plus Gels (10-well) were used to run the samples prepared as shown in 4.2.2.3. The volume of each sample that was added to each well was 30 µL. In the first well, 5 µL of SeeBlue^{TM} Plus2 pre-stained protein standard (10 proteins, 4 - 250 kDa molecular weight markers) was added to monitor protein migration and to serve as a protein ladder to identify the size of the proteins of interest. The loaded samples were run at 200 V using NuPAGE^{TM} MES SDS Running Buffer for 25 minutes in Invitrogen mini gel tank. The gel was then removed from the cassette to be prepared for protein transfer in the next step 4.2.2.5.

4.2.2.5 Protein transfer and staining

The gels containing the separated proteins were placed on a nitrocellulose membrane in an assembled iBlot™ 2 Transfer Stack which were then put on the iBlot™ 2 Gel Transfer Device to transfer the proteins from the gel to the nitrocellulose membrane. The recommended program for Bolt™ 4-12 % Bis-Tris Plus Gels was used (P0, 20 - 25 V, 8 - 10 minutes). The iBlot™ western blotting system is a dry blotting system.

The nitrocellulose membrane was blocked using 5 % w/v BSA in TBS-Tx100 on a rocker at room temperature for 1 h. The primary antibody of the protein of interest was diluted in 5 % w/v BSA in TBS-Tx100 according to supplier instructions and added to the membranes. The details of the used antibodies are mentioned in Table 4.1. The membranes were placed on a rocker in a cold room overnight. Then, the membranes were briefly washed once and then on a rocker for 10 minutes using TBS-Tx100 to remove the residual primary antibodies. The HRP-conjugated secondary antibody was diluted and added to the membranes for 1 h at room temperature on the rocker, which was then followed by 10 minutes wash using TBS-Tx100.

Table 4.1. Antibodies used for western blot. All antibodies were purchased from Abcam (Cambridge, UK).

Antibody	Dilution	Description	Cat No.
Anti-cIAP1	1:250	Rabbit monoclonal	ab108361
Anti-DR5	1:500	Rabbit monoclonal	ab199357
Anti-beta Actin	1:200	Rabbit monoclonal	ab115777
Anti-Rabbit IgG (secondary)	1:2000	Goat HRP conjugated	ab7090

4.2.2.6 Detection

Pierce ECL Western Blotting Substrate (Thermo Scientific, UK) was used on the membranes as a horseradish peroxidase (HRP) substrate for enhanced chemiluminescence (ECL). Chemiluminsence images were immediately captured using the GeneGnome - Syngene bio imaging with GeneSys V1.4.1.0 software. The images were then analysed using densitometry via the Image Studiosoftware. Band densities were normalised against the beta-actin, internal or housekeeping, control. The graphs represent the percentages of the target proteins of each

dose to the negative control. The negative control was normalised to 100 %. Data presented as n=3 ± SEM.

4.2.3 Statistical analysis

The statistical significance was tested using unpaired t-tests on GraphPad Prism 8. If $P<0.05$, the result was considered statistically significant.

4.3 Results

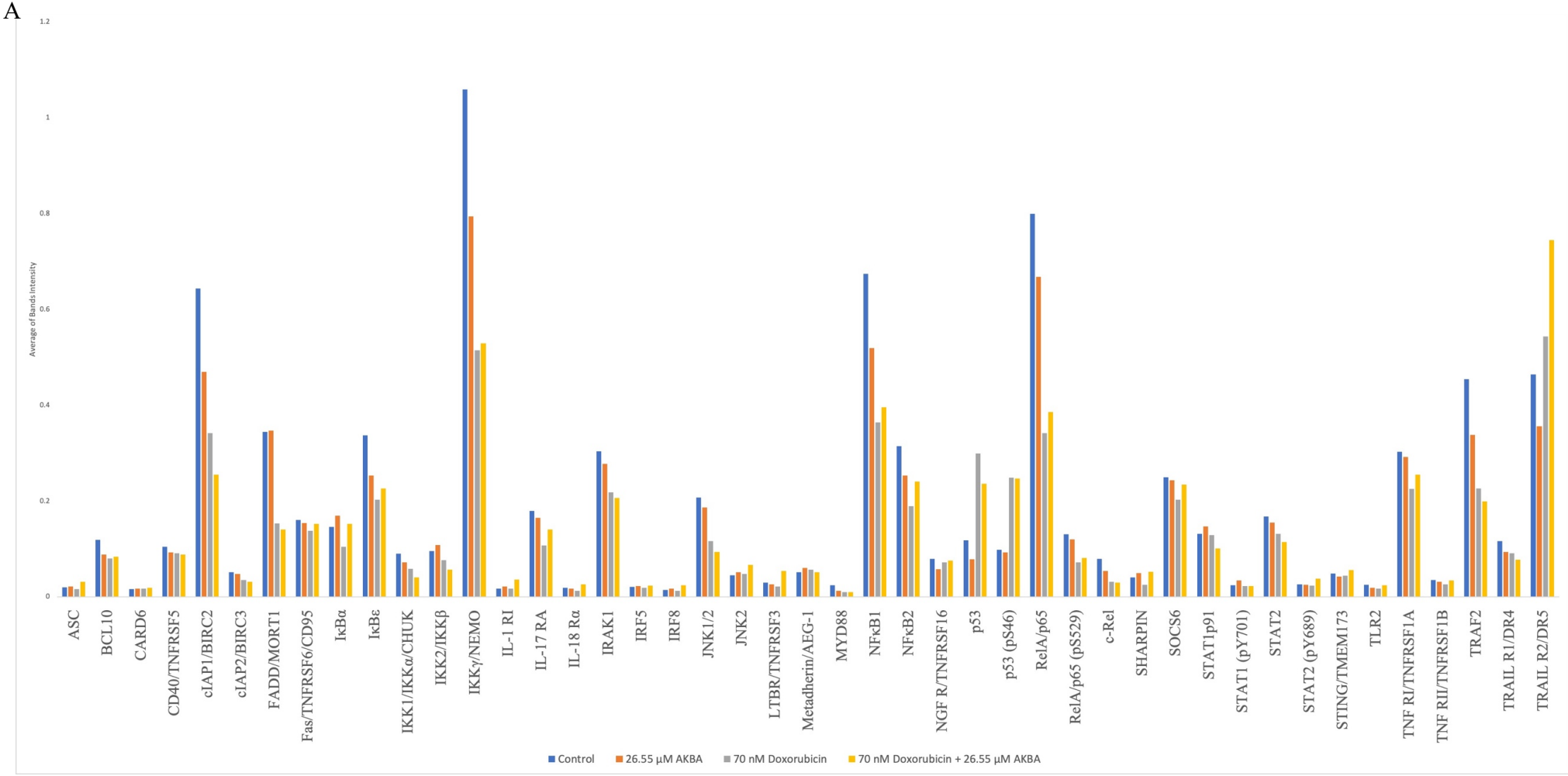
The possible impacts of exposing A2780 cells to AKBA, doxorubicin or both agents added together on NFκB pathway protein expression was studied using Proteome Profiler Human NFκB Pathway Array and then confirmed for selected proteins using western blot. The results showed that treated cells with either AKBA or doxorubicin affected the expression of a number of proteins. Moreover, combining AKBA with doxorubicin changed the effect of doxorubicin on the protein expression in some cases (Figure 4.2). The reduction of the expression of cellular inhibitor of apoptosis protein 1 (cIAP1) was reported as a result of exposing cells to AKBA or doxorubicin. This effect was increased following simultaneous exposure to both compounds. The other noticeable change was on the expression of the death receptor 5 (DR5). Where AKBA decreased the expression of DR5, doxorubicin alone resulted in the increase of DR5. The presence of AKBA improved the effect of doxorubicin on DR5 expression. Therefore, cIAP1 and DR5 were selected for further analysis using western blot.

A2780 cells were exposed to AKBA, doxorubicin and both in combination for 1 and 24 h to confirm the changes on the expression of cIAP1 and DR5 proteins, reported before from proteome array data, using western blot. After 1h of exposure there was no to weak signal of both cIAP1 and DR5 despite the presence of sufficient amount of protein as shown by beta actin (β-actin), the housekeeping or loading protein (data not shown). The expression was apparent after 24 h for both proteins.

The expression of cIAP1 protein was reduced as a result of exposing the cells to AKBA (26 μM) or doxorubicin (1 μM) for 24 h, where the difference was significant ($P<0.001$) compared to the control. The effect of combining AKBA with doxorubicin was negligible and statistically insignificant at this time point despite the apparent difference in the graph (Figure 4.3).

DR5 protein expression was decreased as a result of treating A2780 cells with 26 μM AKBA for 24 h ($P<0.05$), whereas the higher dose of AKBA (62 μM) was not as effective at this time point. In addition, the apparent increase in the expression of DR5 after 24 h treatment with doxorubicin was insignificant. The presence of AKBA with doxorubicin led to a negligible increase in the expression of DR5 at both tested doses of doxorubicin (Figure 4.3).

A



B

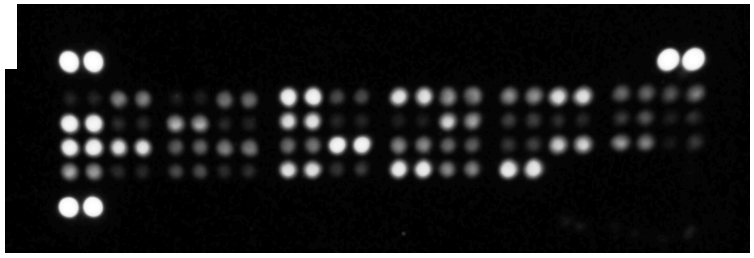


Figure 4.2: The effects of AKBA and doxorubicin alone or combined with AKBA on *NFκB* ***pathway on A2780.*** *Cells were exposed to AKBA, doxorubicin and both combined together for 24 h then Proteome Profiler Human NFκB Pathway Array was used to detect the impact of the tested agents on NFκB pathway. The expression of proteins is shown n=1 (A). The membrane of the control sample after imaging is shown as an example of analysed data (B)*

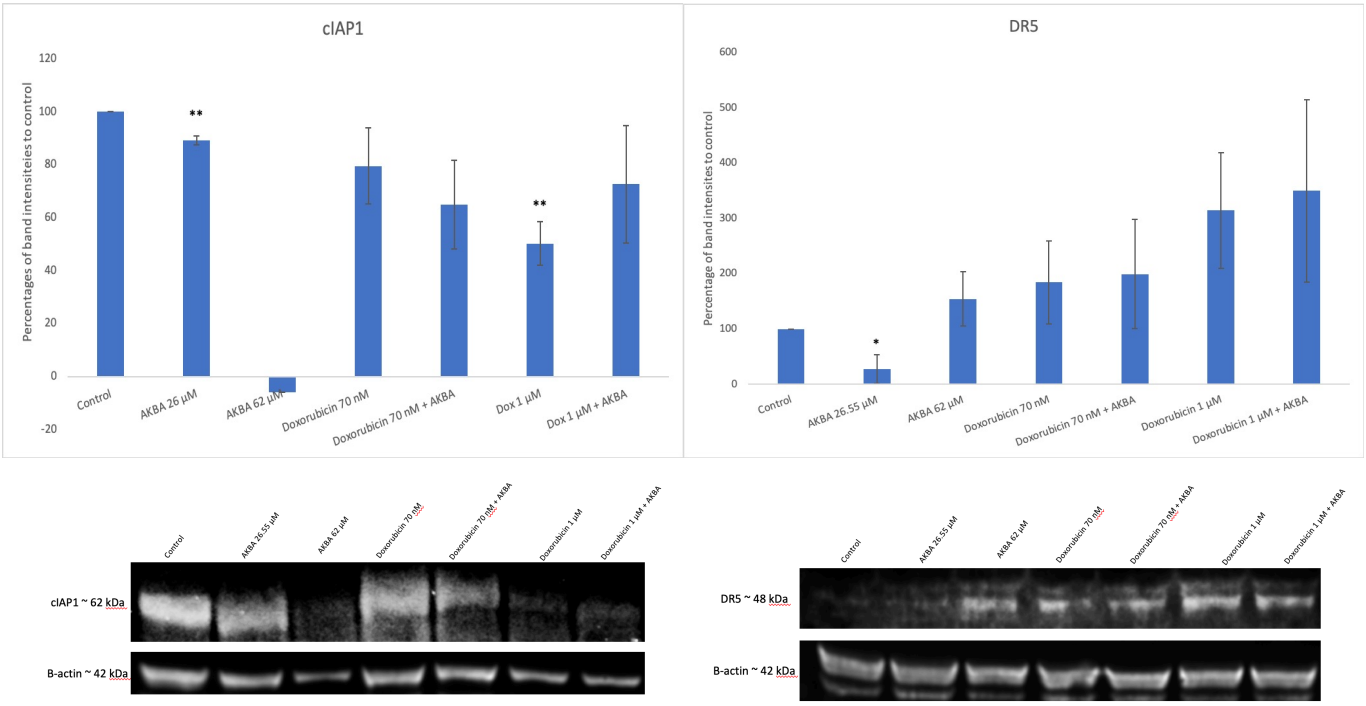


Figure 4.3: cIAP1 and DR5 expression after exposing A2780 cells to AKBA, doxorubicin or the combination of both. *Cells were treated for 24 h. Data is shown as n=3 independent experiments ± SEM. AKBA 62 µM is shown as n=2 therefore error bar is missing. *P<0.05 and **P<0.01 compared to the control.*

4.4 Discussion

The role of NFκB pathway in both inflammation and cancer provides the rationale behind targeting the pathway in cancer therapy. The constitutive activation of NFκB is found in a number of cancers including ovarian cancer, which contributes to the survival, escaping apoptosis and relatively rapid cellular growth in cancers. The NFκB pathway composite proteins are also involved in a number of cellular processes and not limited to the pathway itself. In this chapter, the impact of AKBA, doxorubicin and both combined on the NFκB pathway was investigated and will be discussed.

The common trend that was reported in this project is the reduction of the expression of a number of proteins in the NFκB pathway as a result of the exposure to selected doses of AKBA, doxorubicin or a combination of both agents. Transcription factors of NFκB remain inactive in the cytoplasm due to its binding to IκB-α (NF-kappa-B inhibitor alpha), a family member of inhibitors of NFκB (IκB) (Potz *et al.*, 2017). The expression of IκB-α protein was slightly increased in the cells exposed to AKBA alone. In contrast, doxorubicin exposure led to the reduction of the expression of IκB-α, which was interestingly normalised again after combining AKBA with doxorubicin. The other tested member of IκB family is IκB-ε, which is complexed predominantly to RelA and c-Rel along with IκB- β (Whiteside *et al.*, 1997). The expression of IkB-ε in the treated cells was low compared to the untreated control. These proteins may be contributing to inhibition of NFκB signalling.

The observed changes in the expression of examined members of IκB family brings the inhibitor of nuclear factor kappa-B kinase (IKK) family under the scope. IKK is a multicomponent protein complex (IKK-α, IKK-β and IKK-γ/NEMO) that is responsible for the inactivation of IκB in response to stimuli. IKK inactivates IκB via phosphorylation causing protein degradation which leads to the release of NFκB (Potz *et al.*, 2017). The expression of IKK-α and IKK-γ was reduced in the cells exposed to AKBA alone, whereas IKK-β expression was slightly increased. This confirms results by Syrovets *et al.* (2005) where the inhibition of NFκB by AKBA was linked to its direct inhibitory effect on IKK. The other related observations in the current project are the reduced expressions of IKK-α, IKK-β and IKK-γ in the cells exposed to doxorubicin alone and further reduction of IKK-α and IKK-β in the presence of AKBA was reported.

B-cell lymphoma/leukemia 10 (BCL10) is one of the known regulators of apoptosis and activators of NFκB (Wang *et al.*, 2001; Zhou *et al.*, 2004). The expression of BCL10 was

reduced in the cells treated with AKBA, doxorubicin and both agents combined. BCL10 reduction (Karin, 2006) combined with overall inactivation of NFκB pathway may enhance the probability of apoptosis contributing to the reported synergism between AKBA and doxorubicin.

Fas (CD95), a prototypic death receptor, induces apoptosis and activates NFκB through fas-associated protein with death domain (FADD), which induce NFκB activity via caspase 8 and RIP (receptor-interacting protein kinases) (Kreuz *et al.*, 2004). The expression of both Fas and FADD was not affected by exposing cells to AKBA. This agrees with the findings that were reported by Liu *et al.* (2002) where AKBA induced apoptosis in colon cancer HT-29 cells through caspase 8 in a pathway that was independent on Fas/FasL interaction. They state that expression of Fas was not changed when cells treated with AKBA. While Fas expression was slightly reduced after exposing the cells to doxorubicin, FADD expression was almost halved compared to the untreated control. Similar observations on breast cancer cells MCF-7 were reported (Liu and Chang, 2011).

Members of inhibitor of apoptosis (IAP) protein family have been targeted to treat cancer by developing agents that inhibit their function to serve as a promising approach to induce apoptosis in cancer cells (Straub, 2010). Changes in IAPs contribute to chemoresistance, disease progression and poor prognosis of a number of cancers. In addition to their primary function in regulating caspases, IAPs are involved in other cellular processes including the activation of NFκB transcription factors (Gyrd-Hansen and Meier, 2010). Based on the proteome analysis results that was then confirmed by western blot, cIAP1 protein expression was down-regulated by AKBA (26 μM) and doxorubicin (1 μM). Similar but less noticeable impacts were reported on cIAP2. Roy *et al.* (2016) state that IAP1 is one of the molecular targets of boswellic acids. Combining AKBA with doxorubicin has not affected the inhibitory effect of doxorubicin on cIAP1. Even though there was no significant change in the effect of doxorubicin on cIAP1 when combined with AKBA compared to doxorubicin alone, more time points and a wider range of doses may lead to a more conclusive data.

Interleukin 17 receptor A (IL-17 RA) is a membrane protein that binds to interleukin 17A (IL-17A). NFκB is one of the downstream targets of IL-17A in many cells (Gu, Wu and Li, 2013). IL-17A increased the expression of MMP2 and MMP9 via NFκB activation, which was linked to promoting metastasis in hepatocellular carcinoma (Li *et al.*, 2011). While the expression of IL17 RA was slightly reduced by AKBA, it was halved by doxorubicin. In the

presence of AKBA, the reduction in expression of IL-17RA induced by doxorubicin was largely counteracted.

One of the upstream activators of NFκB is interleukin-1 receptor-associated kinase 1 (IRAK1), the first discovered member of interleukin-1 receptor-associated kinases (IRAKs). IRAKs are important in a number of normal and pathological cellular processes including cellular differentiation, apoptosis and inflammation. To activate NFκB, Phosphorylated IRAK1 binds to E3 ubiquitin ligase (TRAF6) (Rhyasen and Starczynowski, 2015). IRAK1 could be a target to prevent metastasis, resistance and reduce the size of solid tumours, where it was found overexpressed in various types of cancers and contributes to tumorigenesis (Zhang *et al.*, 2014; Wee *et al.*, 2015; Yang *et al.*, 2019). In this study, the expression of IRAK1 was reduced in the cells treated with AKBA and doxorubicin alone. Further reduction in the expression of IRAK1 was observed when the two agents were combined suggesting reduced NFκB phosphorylation and therefore reduced NFκB signalling.

The following NFκB transcription factors: NFκB1, NFκB2, RelA (and Rela pS529) and c-RelA expressions were examined in this NFκB proteome analysis after exposing cells to the tested agents for 24 h. Their expression was reduced when cells were treated with AKBA, doxorubicin or both combined compared to the untreated control. The presence of AKBA with doxorubicin slightly reduced the effect of doxorubicin on the transcription factors even though it remained lower than the control. Changes in the expression of NFκB1 and RelA were more pronounced than the other factors. This is in line with the other observations discussed above where a general inhibition of NFκB was shown.

The participation of p53 in a broad range of important cellular events, such as cell cycle regulation, DNA repair and apoptosis induction, and for the fact that p53 abnormality is widely found in various types of cancers makes it one of the important proteins to explore and understand (Zhou, Hao and Lu, 2019). Mutant p53 contribution to tumour survival and invasion through TNFα-dependent activation of NFκB is one of the examples that explains the involvement of p53 in NFκB pathway (Mantovani, Walerych and Sal, 2017). In this project, it was reported that AKBA reduced the expression of p53 while doxorubicin increased its expression and the expression of the phosphorylated p53 (pS46). Interestingly, the impact of doxorubicin on p53 expression was reduced by AKBA. This could be used as an indicator that AKBA induced cell cycle arrest and apoptosis in a p53-independent manner (see section: 1.2.2).

TRAF2 (tumour necrosis factor receptor (TNFR)–associated factor (TRAF)) belongs to the TNF receptor-associated factor (TRAF) family and it is one of the activators of NFκB (Zhang *et al.*, 2011). The expression of TRAF2 was decreased in the cells treated with AKBA or doxorubicin. Moreover, combining AKBA with doxorubicin led to further reduction in the expression of TRAF2. These observations confirm the other discussed data where a down-regulation of a number of NFκB activators was reported.

Death receptor 5 (DR5, TRAIL R2 (tumour necrosis factor-related apoptosis-inducing ligand receptor 1)) expression is regulated by NFκB among other regulators. Activated NFκB up-regulates the expression of the DR5 by cooperating with p53, where both proteins induce apoptosis (Shetty *et al.*, 2005). DR5 expression was first tested using the NFκB proteome analysis then was selected to be the second candidate to confirm using western blot. AKBA reduced the expression of DR5 when used at 26 μM for 24 h, which was relatively non-toxic. On the other hand, the expression of DR5 was not significantly changed in the cells exposed to doxorubicin neither in the cells exposed to doxorubicin combined with AKBA (26 μM). Similar to cIAP1, the effect of different agents on the expression of DR5 needs more exploration.

To sum up, AKBA and doxorubicin alone or combined at the tested doses and time points showed the ability to downregulate NFκB using the NFκB proteome analysis. Considering the synergistic interaction between the agents shown in previous chapters, NFκB could be one of the shared targets through acting on different proteins in the pathway. This impact on the NFκB pathway needs more investigation using other time points and wider range of the concentrations, which could provide more information on the involvement of NFκB on the interaction between AKBA and doxorubicin. For example, DR5 and cIAP1 protein expression were triggered sometime after 1 h and before 24 h from the exposure, which can be seen from the absence of the signal at 1 h. The other suggested approach to tackle limitations in this project is to repeat the NFκB proteome analysis at least twice to apply a more robust statistical analysis on the data. Moreover, NFκB activity, rather than expression, could be monitored overtime using a technique described by Badr *et al.* (2009) to understand the role of inhibition or activation of NFκB on the therapeutic effect of the agents.

Chapter 5

Nuclear Factor E2-related Factor 2 (Nrf2) expression, Reactive Oxygen species (ROS) generation and Permeability glycoprotein (P-gp) expression

5 Nuclear Factor E2-related Factor 2 (Nrf2) and Reactive Oxygen species (ROS)

5.1 Introduction

5.1.1 Nuclear Factor E2-related Factor 2 (Nrf2) and Reactive Oxygen species (ROS)

Exogenous, e.g. UV or drugs, and endogenous, e.g. ROS, stresses could threaten the normal functionality of cells, leading to DNA damage, cell destruction or could induce tumorigenesis. A number of molecular components could be involved in the cellular defence mechanisms. The key defensive pathway against oxidative and electrophilic stress is Keap1-Nrf2 signalling pathway. Nrf2 is normally guarded by actin-associated Keap1 protein in the cytoplasm. Keap1 releases Nrf2 in response to the stress signalling, which in turn move to the nucleus and binds to a *cis*-acting enhancer called antioxidant-response element (ARE). This binding regulates the ARE-mediated antioxidant enzyme gene expression of a variety of enzymes involved in cellular detoxification processes, e.g GSTA2 (glutathione S-transferase A2), NQO1 (NADPH: quinone oxidoreductase 1) and HO-1 (Heme Oxygenase 1) (Lee and Johnson, 2004; Kaspar, Niture and Jaiswal, 2009; Nguyen, Nioi and Pickett, 2009). The main function of this mechanism is maintaining the redox balance in cells, which helps in the cell survival.

Nrf2 cytoprotective ability is linked to cancer chemoprevention in normal and premalignant cells. A variety of phytochemicals, extracted from different plants, with potent chemo-preventive activities were found to be Nrf2 inducers. However, Nrf2 could also be part of tumorigenesis and cancer progression. It could be involved in chemoresistance by protecting cancer cells from the chemical stress caused by chemotherapeutic agents (Lau *et al.*, 2008; Kansanen *et al.*, 2013). One of the ways that Nrf2 contributes to chemoresistance is the regulation of efflux transporters such as p-glycoprotein (P-gp) (Jeddi *et al.*, 2017; Sadeghi *et al.*, 2018). This dual role seems to be controlled by the microenvironment of the cell. A number of target proteins are regulated by Keap1-Nrf2 signalling pathway (Table 5.1).

In this chapter, the effects of the tested agents on Nrf2 expression are examined in an attempt to explore if it is one of the possible reasons of the apparent antagonism between AKBA and cisplatin. The other part of this chapter focuses on the possible impacts of the agents on ROS generation which plays important role in tumorigenesis.

Table 5.1: Key target proteins in Keap1-Nrf2 signalling pathway categorisation (Jeddi *et al.*, 2017).

Antioxidants	Detoxification enzymes	Membrane transporters
Heme oxygenase-1 (HO-1) Thioredoxin 1 (Trx1) Thioredoxin reductase (TrxR) Aldo–keto reductases (AKR) Glutathione peroxidase (GPx) Sulfiredoxin 1 (Srx1) peroxiredoxin 1 (Prx1) Glutathione (GSH) Glutamate cysteineligase (GCL) Glucose-6-phosphate dehydrogenase (G6PD) NAD(P)H: quinine oxidoreductase 1 (NQO1) Ferritin heavy (FTH1) Ferritin light (FTL) chains	Glutathione S-transferases (GSTs) Uridine 5′-diphospho-glucuronosyltransferase(UGT)	ATP-binding cassette sub-family F member 2 (ABCF2) Multidrug resistance-associated proteins (MRPs) P-glycoprotein (P-gp/MDR1/ABCB1) Breast cancer resistance protein BCRP/ABCG2

5.1.2 Permeability glycoprotein (P-gp) expression

As discussed in the introduction of this thesis, drug efflux from cancer cells is one of the mechanisms behind multidrug resistance (MDR). One of the molecular events that could lead to the efflux is the overexpression of MDR-1 gene, also called ABCB1 gene, which encodes an ATP cassette transporter protein called permeability glycoprotein (P-gp). In addition to the primary physiological role of P-gp by helping cells to remove toxins and xenobiotics through

the plasma membrane, it is overexpressed in many cancer types, including ovarian cancer, and involved in other diseases such as Alzheimer's disease. The structure of P-gp allows it to react with a broad range of sizes of cationic amphipathic molecules. P-gp is 170 kDa composed of two pseudo-symmetrical halves (Gottesman and Pastan, 2015; Katayama *et al.*, 2015; McCormick, Vogel and Wise, 2015).

P-pg overexpression in chemoresistant cancer cells causes drug efflux leading to the reduction of the intracellular concentration of therapeutic agents and their cytotoxicity. Doxorubicin is one of a wide spectrum of antitumor agents that could be affected by the expression of P-gp in tumour cells. Considering its importance in MDR, targeting P-gp and its substrates using synthetic agents or natural product extracts is one of the emerging approaches to overcome drug resistance in cancer treatment (Raghava and Lakshmi, 2012; Abdallah *et al.*, 2015).

The synergistic interaction between AKBA and doxorubicin was reported earlier in this project (see chapter 2). Establishing the effects of each of the agents alone or both combined on P-gp could help in revealing one of the molecular targets of the agents or could provide an explanation of the interaction between them.

5.2 Methods

5.2.1 Western blotting for Nrf2

The western blot procedure mentioned in 5.2.2 was followed. The antibodies used here are listed in Table 5.2.

Table 5.2. The antibodies. All antibodies were purchased from Abcam.

Antibody	Dilution	Description	Cat No.
Anti-Nrf2	1:1000	Mouse monoclonal	ab89443
Anti-HO-1	1:500	Mouse monoclonal	ab13248
Anti-beta Actin	1:250	Mouse monoclonal	ab8226
Anti-Rabbit IgG	1:2000	Rabbit HRP conjugated	ab6728

5.2.2 Reactive oxygen species (ROS) assay

A quantitative microplate assay for ROS detection was used to assess the effect of AKBA, doxorubicin, cisplatin and AKBA combined with either doxorubicin or cisplatin on ROS generation on A2780. In this assay, ROS activity in cells is measurable by using a fluorogenic dye, 2',7' –dichlorofluorescin diacetate (DCFDA). Cellular esterases deacetylate DCFDA to a non-fluorescent compound, which is retained in the cell. The compound can then be oxidised by various oxidising species ROS to produce 2', 7' –dichlorofluorescein (DCF), which is highly fluorescent.

Cells were seeded at 4 x 10^5 cells per well in black 96-well plates with clear bottom in a phenol red free RPMI-1640 +10 % NCS. Cells were incubated at 37 °C in 5 % CO_2 for 24 h to attach and acclimatise. The medium was removed and the cells were washed once with PBS. The cells were then incubated with with 20 μM DCFDA (Catalogue Number: D399, Fisher, UK) in PBS for 45 minutes at 37 °C in 5 % CO_2. The cells were then washed once with PBS to remove non-absorbed DCFDA. Fresh PBS was added with various concentrations of the agents and cells exposed the cells for 1 h, at 37 °C in 5 % CO_2. Fluorescence signal was monitored at excitation/emission (475/500-550) using a microtiter plate reader (GloMax® Discover System, GM3000, Promega Corporation, USA).

For 24 h exposure, phenol red free medium + 10 % NCS was used with the agents instead of PBS after allowing cells to acclimatise and staining them with DCFDA. Cells were then

incubated at 37 °C in 5 % CO_2 for 24 h with selected doses of the tested agents. The medium was then removed and cells were washed once with PBS. Finally, fresh PBS was added and the plates were taken to quantify the fluorescence at excitation/emission (475/500-550) using the microtiter plate reader (GloMax® Discover System, GM3000, Promega Corporation, USA). The background, that may result from fluorescent compounds in the cells or culture medium, was subtracted using unstained cells as baseline of the experiment. The percentages of each dose of the negative (untreated) control was calculated using Excel.

5.2.3 Western blotting for Pgp

The western blot procedure mentioned in 5.2.2 was followed. The antibodies used here are listed in Table 5.3. The reagents used in this chapter are AKBA (26 and 62 µM) and doxorubicin (70 nM and 2 µM). A2780 cells were exposed to the agents separately or combined for 1 and 24 h.

Table 5.3: The antibodies. All antibodies were purchased from Abcam

Antibody	Dilution	Description	Cat No.
Anti-Pgp	1:500	Rabbit monoclonal	ab170904
Anti-beta Actin	1:200	Rabbit monoclonal	ab115777
Anti-Rabbit IgG	1:2000	Rabbit HRP conjugated	ab6728

5.2.4 Flow cytometry

After treating A2780 cells with according to the planned approach, samples were run in BD Accuri C6 plus flow cytometer. The protocol provided by Abcam was followed (See: https://www.abcam.com/protocols/direct-flow-cytometry-protocol). Mouse monoclonal [UIC2] to P Glycoprotein (Phycoerythrin) (ab93590, Abcam) was used to determine the expression of P-gp. Mouse monoclonal IgG2a (ab91363) was used as an isotype control. Collected data were analysed using FlowJo (V10, USA). One experiment was performed because of the limited access to the flow cytometer.

5.2.5 Statistical analysis

The statistical significance was tested using unpaired t-tests on GraphPad Prism 8. If $P<0.05$, the result was considered statistically significant.

5.3 Results

5.3.1 Nrf2 expression modulation

The effect of AKBA, cisplatin, doxorubicin or either combined with AKBA on Nrf2 protein expression on A2780 cell was examined at two different time points 1 and 24 h of exposure using western blot (Figure 5.1). Heme Oxygenase 1 (HO-1) protein expression was used as an indicator of Nrf2 activity, where Nrf2 activation activates the expression of HO-1 among other cellular defence genes.

The expression of Nrf2 was not affected after exposing cells to AKBA for 1 h neither for 24h. HO-1 expression was slightly elevated at 26.55 µM and downregulated at 62 µM AKBA in the cells exposed for 1 h.

The second approach considered exposing cells to cisplatin or cisplatin combined with 26 µM AKBA. There was a reduction in the expression of both proteins after 1 h of the exposure to cisplatin. The reduction was significantly different to the control. The presence of AKBA did not change the effects of cisplatin on Nrf2 and HO-1. The results of 24 h exposure showed negligible/no difference in the expression of Nrf2 and HO-1 in the cells exposed to cisplatin compared to the contol or cisplatin combined with AKBA compared to the control and cisplatin alone.

The final approach was by treating cells with doxorubicin alone or combined with AKBA for 1 or 24 h. Similar to cisplatin, a reduction in the expression of both Nrf2 and HO-1 proteins was reported after 1 h of the treatment. The presence of AKBA did not affect the effect of doxorubicin on both proteins. Following 24 h exposure, Nrf2 level of expression was similar to the control at 62 nM, whereas a significant increase was noted at 2 µM. A statistically significant increase in Nrf2 production was reported as a result of the presence of AKBA with doxorubicin compared to doxorubicin solely at both doses. On the other hand, significant downregulation of HO-1 was observed following the combination of AKBA with 2 µM doxorubicin compared to the control, while doxorubicin alone slightly increased HO-1 expression.

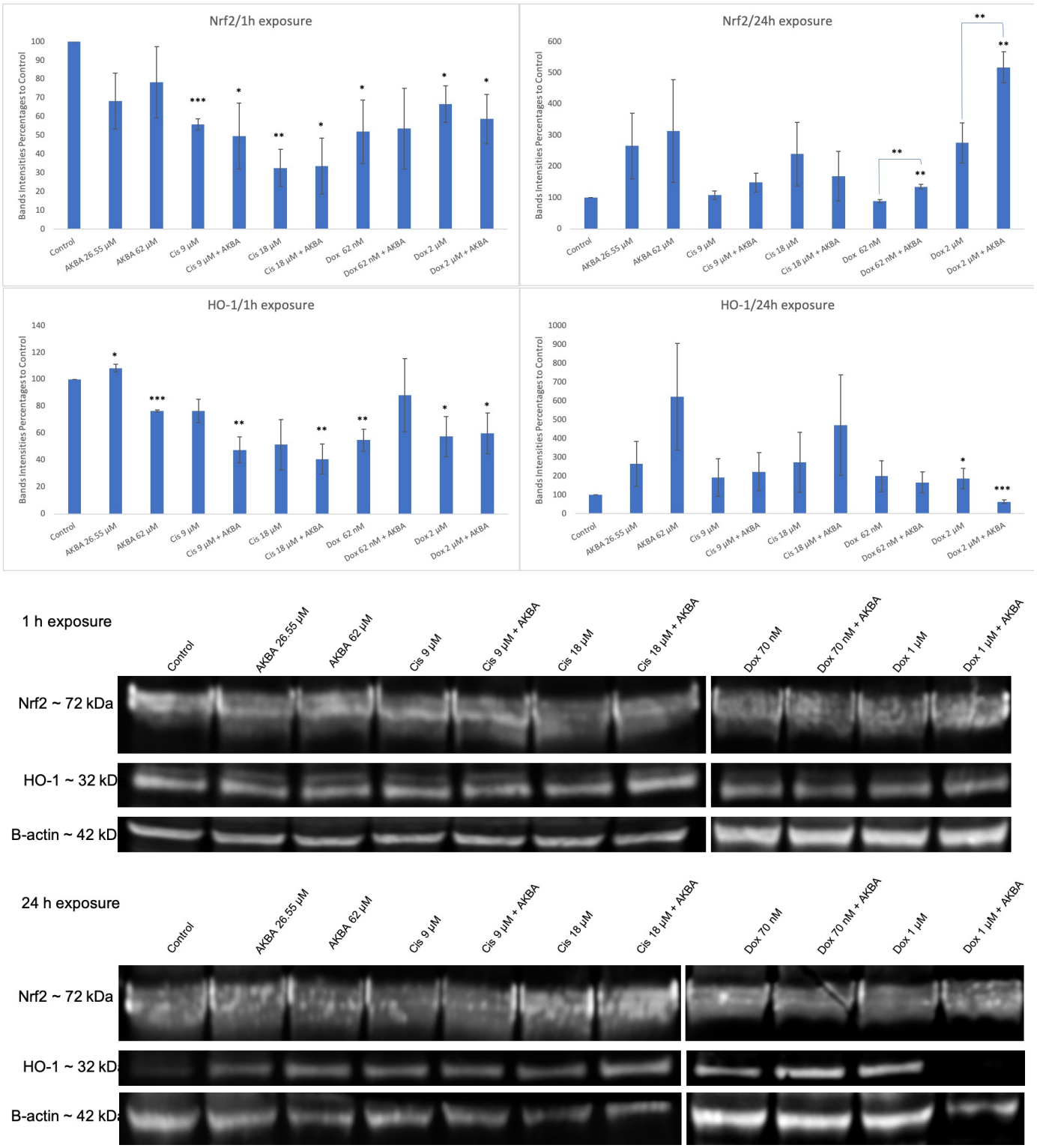


Figure 5.1: Nrf2 and HO-1 expression following the exposure of A2780 to AKBA, cisplatin, doxorubicin or either combined with AKBA for 1 and 24 h. *Cis = cisplatin and Dox = doxorubicin. The expression of the proteins was determined using western blot. Data represents at least 3 independent experiments ± SEM. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 compared to the control. **P<0.01compared to the same dose in the absence of AKBA.*

5.3.2 Reactive oxygen species (ROS) generation

The impact of various treatment approaches used in this project on ROS generation in A2780 cells was investigated using DCFDA, a fluorogenic dye (Figure 5.2). Cells were treated with the tested agents for 1 or 24 h. Exposing the cells to AKBA for 1h resulted in a significant reduction in ROS levels, whereas ROS generation was similar to the untreated cells after 24 h. Similar observations where reported following the exposure to different concentration of cisplatin for 1 and 24 h. The effect of cisplatin on ROS formation was not significantly changed as a result of the presence of AKBA at both time points. Finally, the levels of ROS were not affected after treating the cells with doxorubicin for 1 h. In contrast, ROS levels were reduced in a dose dependent manner after 24 h of treating cells with doxorubicin. There was no reported changes to the effects of doxorubicin on ROS after combining it with AKBA for 1 or 24 h.

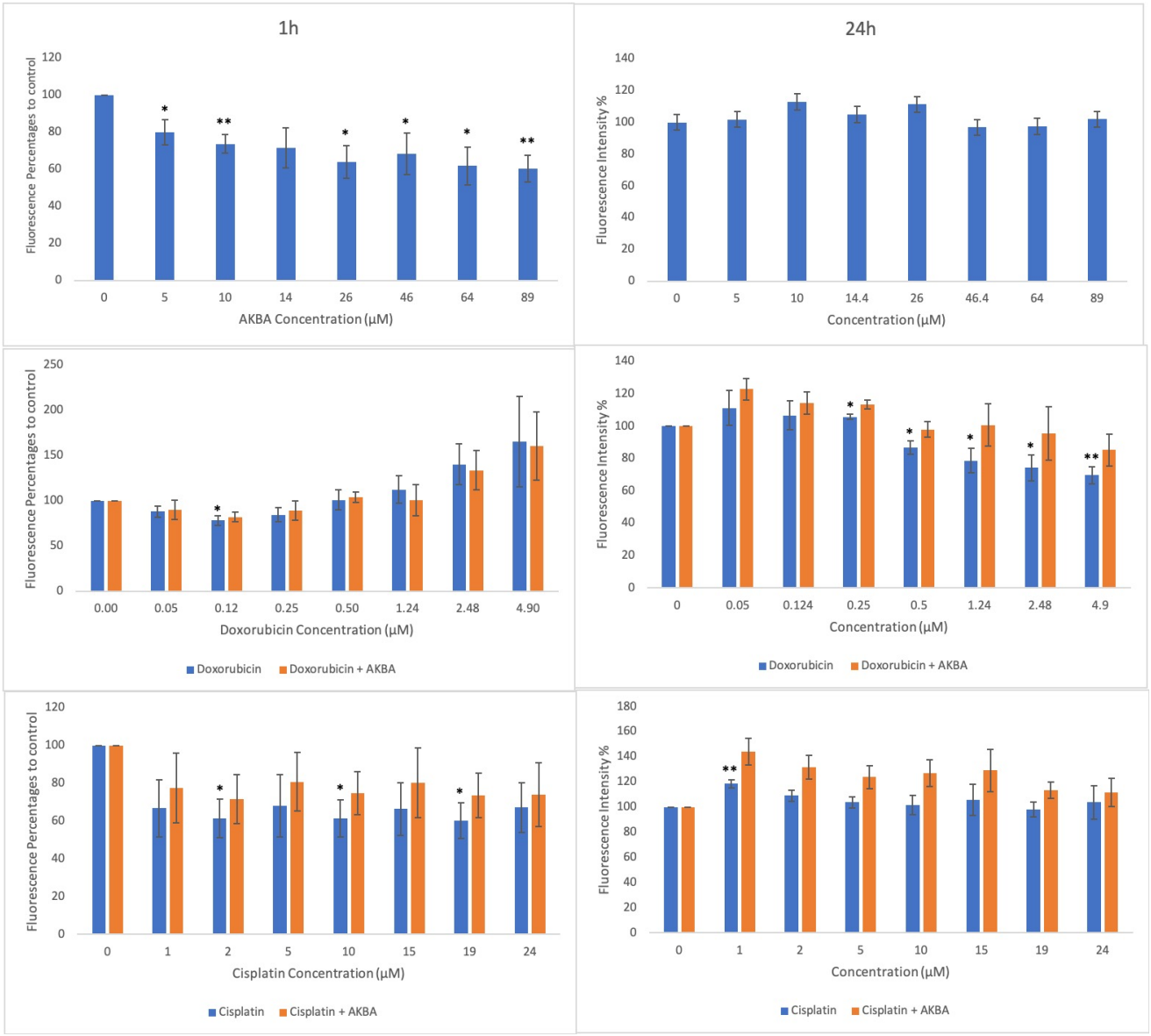


Figure 5.2: ROS generation in A2780 cells following the exposure to AKBA, cisplatin, doxorubicin or either of the chemotherapies combined with AKBA. *The levels of the ROS were determined using DCFDA as described in the methods section. Data represents mean n= 3 independent experiments ± SEM. *P<0.05 and **P<0.01.*

5.3.3 P-gp expression modification by AKBA and doxorubicin

The impact of AKBA, doxorubicin and both agents combined on P-gp expression in A2780 was studied using western blot. The cells were treated with selected doses of the agents for 1 and 24 h. Collected cell lysates were run and band intensities were calculated. The expression of P-gp was not altered after 1 h of exposing cells for both doses of AKBA (26 and 62 μM). A significant increase in the expression of P-gp was reported at 62 μM AKBA after 24 h of the exposure (Figure 5.3).

The expression of P-gp in the cells exposed to doxorubicin at 2 μM for 1h was significantly decreased compared to the untreated cells, while the change of P-gp expression in cells exposed to 70 nM doxorubicin was negligible at both time points. It seemed that P-gp expression is higher than the control in the cells exposed to 2 μM doxorubicin for 24 h (P=0.07). More repeats of the experiment may reveal a significant effect. The presence of 26 μM AKBA with both doses of doxorubicin has shown no impact on the effects of doxorubicin on P-gp expression at both time points 1 and 24 h (Figure 5.3).

5.3.4 Testing the effects of selected agents on P-gp expression using flow cytometry

The flow cytometry analysis of the expression of P-gp will be discussed here. There was an increase in P-gp expression after exposing A2780 cells to AKBA. However, there was a slight reduction in the expression of P-gp as a result of treating the cells with doxorubicin. Further reduction in the expression was reported when AKBA was combined with doxorubicin at both doses 70 nM and 2 μM. More experiments are required to confirm these results (Figure 5.3, C).

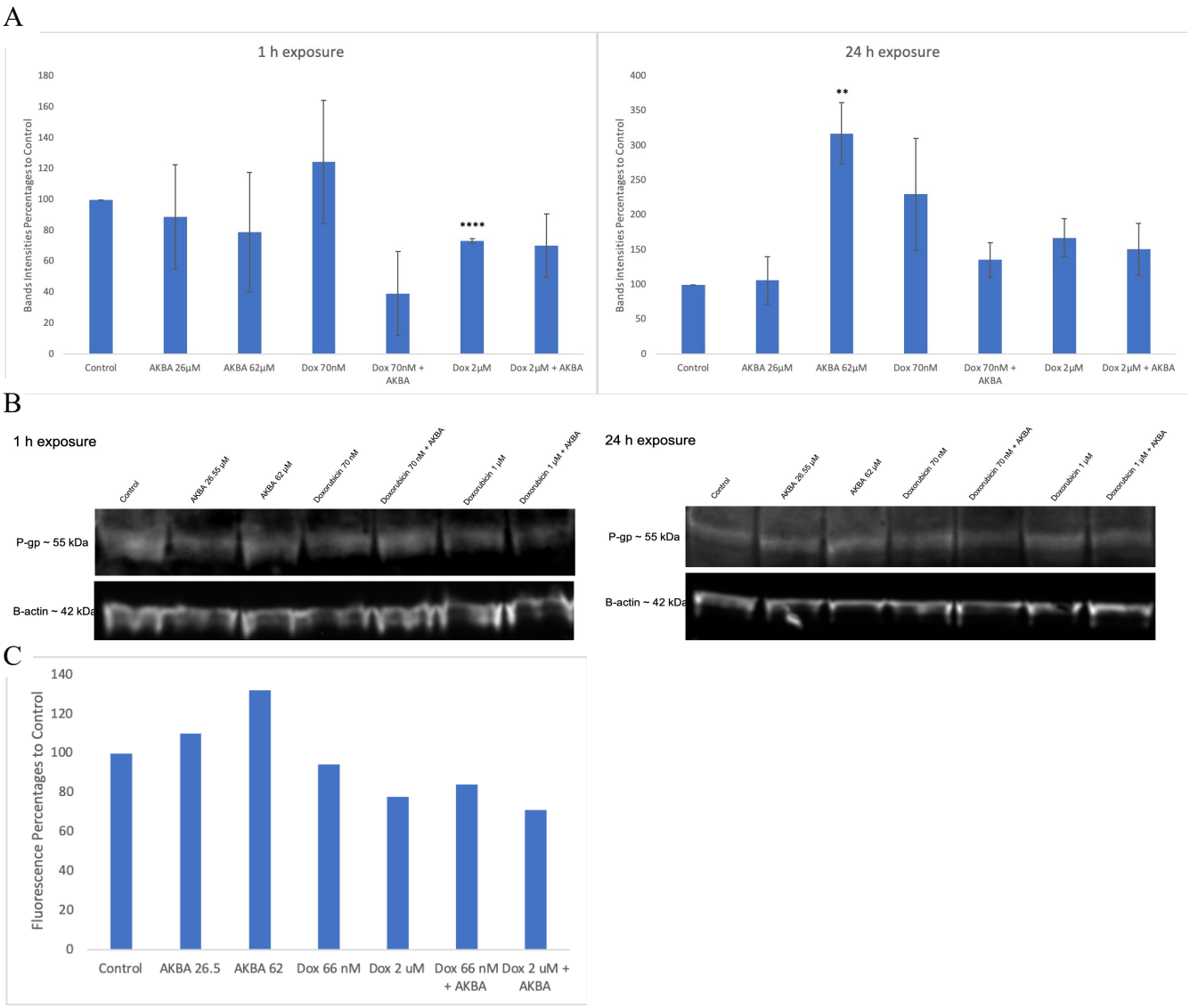


Figure 5.3. The expression of P-gp in A2780 exposed to AKBA, doxorubicin and a combination of both agents for 1 and 24h. *Cells were treated with the selected doses for 1 and 24 h. The percentages of the expression of P-gp in A2780 compared to control cells are shown as three independent experiments ± SEM (A). B shows the bands on the membrane after running samples using western blot. The graph (C) shows the percentages of fluorescence of various doses compared to untreated cells (n=1) following the exposure of cells to the agents for 24 h.*

5.4 Discussion

5.4.1 Nuclear Factor E2-related Factor 2 (Nrf2) and Reactive Oxygen species (ROS) Generation

In normal cells the transcription factor Nrf2 plays a key role in in the cellular defence mechanism against ROS and other potentially toxic agents to maintain the redox haemostasis, which is necessary for cells health. Nrf2, however, can be used by cancer cells to survive various endogenous and exogenous stresses through inducing the expression of about 500 genes that encode proteins with antioxidant and cytoprotective functions. HO-1 is a particular protein that helps cancer cells to grow and resist chemotherapies due to its antioxidant and antiapoptotic effects. It was reported that Nrf2 and HO-1 are overexpressed in various cancer types including ovarian carcinoma (Furfaro *et al.*, 2016). The influence of AKBA, cisplatin, doxorubicin or either of the conventional chemotherapies combined with AKBA on Nrf2, HO-1 and ROS generation was examined in this chapter. The main purpose of this exploration was studying the possible molecular pathways involved in the antagonistic interactions between cisplatin and AKBA.

Induction of Nrf2/HO-1 defence mechanism by AKBA is one of the suggested reasons behind the antagonistic interaction between cisplatin and AKBA. It was reported that Nrf2 is one of the factors that induce cisplatin resistance in ovarian cancer cell (Bao *et al.*, 2014; Wu *et al.*, 2017).. However, Nrf2 expression was only reduced by cisplatin after 1 h, whereas its expression was similar to the untreated cells in the cells exposed to cisplatin for 24 h and/or AKBA for 1 h and 24 h. Although the results in this project do not support the involvement of Nrf2/HO-1 pathway in the reported antagonistic interaction between AKBA and cisplatin, more time points and a wider range may reveal a more conclusive outcomes. The other way that may help in building a better understanding is measuring the effect of the tested agents on the activity of Nrf2 rather than the protein expression.

In addition, it was stated that AKBA cytoprotective effect against doxorubicin induced hepatotoxicity may occur due to the antioxidant activity of AKBA that might happen through modulation of the Nrf2/HO-1 pathway (Barakat *et al.*, 2018). This published evidence could raise questions about the reported synergistic interaction between AKBA and doxorubicin despite the increased expression of Nrf2 in the cells exposed to doxorubicin in the presence of AKBA compared to the control and cells exposed to doxorubicin alone for 24 h. The proposed answer to this argument is the reduced expression of HO-1 in the cells treated with

both agents together, which could be used as a way to measure Nrf2 activity. The other speculated explanation is the molecular differences between normal and cancer cells. Therefore, it is reasonable to assume that AKBA, doxorubicin or Nrf2 function differently in different environments. It is also logical to say that there are other molecular targets that lead to the synergistic interaction between AKBA and doxorubicin such as NFκB, which in turn could contribute to overcoming triggered Nrf2/HO-1 defence mechanisms.

With regards to ROS, cells were able to retrieve the balance of generated ROS after 24 h of exposing them to AKBA or cisplatin, while a reduction of ROS was reported at 1 h in the cells treated with AKBA or cisplatin. The reduced ROS formation by cisplatin at 1 h coincided with down-regulation of Nrf2 and HO-1. This may indicate that the antioxidant activity, at this particular time point and the used doses, is a result of a mechanism other than Nrf2/HO-1. Uttara *et al.* (2009) classified antioxidants based on their mechanism of action into four classes: direct (non-enzymatic), indirect, metabolic and metal containing. Doxorubicin, on the other hand, reduced ROS levels in the cells treated for 24 h in a dose dependent manner.

The antagonistic interaction between AKBA and cisplatin could not be linked to Nrf2/HO-1 pathway in this project. Further experiments are required to explore the possibility of the involvement of the pathway in the antagonistic interaction between AKBA and cisplatin. This could benefit from applying different experiments such as testing the activity of Nrf2 in the treated cells using a more extensive measure of Nrf2 transcriptional activity (Czauderna *et al.*, 2018). In addition, examining various treatment approaches are recommended to avoid the antagonistic interaction. Pre-exposing cells to AKBA for 1 or 2 h before treating cells with cisplatin is one of the suggested approaches considering the increased expression of Nrf2 occurs at some point between 1 and 24 h. According to Sirota, *et al.* (2017), caffeic acid interaction with cisplatin on ovarian cancer models was dependent on the treatment approach. While pre-exposing cells to caffeic acid for 6 h caused the cell to be resistant to cisplatin, exposing them to caffeic acid and cisplatin simultaneously sensitised the cells to cisplatin.

5.4.2 P-gp expression modification by AKBA and doxorubicin

In an attempt to investigate the molecular events that could contribute to the observed synergistic interaction between AKBA and doxorubicin, P-gp protein expression was evaluated in the cells treated with AKBA, doxorubicin or both agents combined.

Overexpressed P-gp is one of the main reasons that give rise to chemoresistance in various types of cancers. In a study by Singh and Lamprecht (2015), nanoparticles loaded with P-gp inhibitors sensitised the resistant cell line A2780ADR to doxorubicin. Consequently, targeting P-gp is one of the promising approaches to overcome drug resistance (Bao *et al.*, 2011; Yamagishi *et al.*, 2013; Syed *et al.*, 2017; Nanayakkara *et al.*, 2018).

The results in this thesis do not support the hypothesis that P-gp is one of the molecular targets when trying to explain the synergistic interaction between AKBA and doxorubicin. The expression of P-gp in the cells treated with doxorubicin alone was lower than the negative control after 1 h and similar to the control after 24 h. This was not changed by combining AKBA (26 μM) with doxorubicin. It could be that the doses and the chosen time points are not enough or the approach of testing needs to be changed.

On the other hand, the high dose of AKBA (62 μM) alone induced the expression of P-gp. This could mean that the impact of AKBA on P-gp is dependent on the dose or the cellular condition, i.e. AKBA modulated the expression of P-gp in favour of the chemotherapy efficacy.

Further confirmative tests are required to support or refute current observations or the hypothesis. One of the tests could be examining the activity of P-gp in response to the tested agents. The other approach could involve comparing the expression and/or activity of P-gp in A2780 to its doxorubicin counterpart A2780ADR. Moreover, the reported impact of AKBA on P-gp expression in A2780 could be investigated using A2780ADR.

Chapter 6

General Discussion, Conclusions and Future Work

6 General Discussion, Conclusions and Future Work

Ovarian cancer is a difficult disease to treat and is usually diagnosed in a late stage. It can metastasise to the surrounding environment in a unique way in the early stages (Lengyel, 2010). Following surgery and chemotherapy, ovarian cancer is able to relapse and develop resistance to first line chemotherapy, which could result from intrinsic or acquired resistance (Pokhriyal *et al.*, 2019). Development of new treatment regimens to treat ovarian tumour is crucial. A large number of the available drugs used to treat cancer were discovered primarily in plants and thus phytotherapeutic compounds may provide a valuable addition to the treatment of this disease. Potential anti-tumour activity of BSE and BAs against various types of tumours is one of the encouraging reasons to explore them in this project.

6.1 AKBA and BSE cytotoxicity towards ovarian cancer cells and possible molecular targets

AKBA cytotoxicity towards ovarian cancer was addressed, for the first time, by AlSalmani *et al.* (2013) as part of a PhD project under the supervision of Dr. Evans, at the University of Leicester. In this thesis we confirm the potential anti-cancer activity against ovarian cancer models using A2780, A2780cis, A2780ADR and OVCAR4 cell lines and investigated its interaction with selected conventional chemotherapies, cisplatin, paclitaxel and doxorubicin, which will be discussed with more details in the next few paragraphs. Furthermore, the cytotoxicity of frankincense extracts were tested on A2780 and A2780cis. The ethanol extracts showed potential anti-cancer activity as evidenced by the its toxicity towards the examined ovarian cancer lines (A2780 and A2780cis), which agrees with published literature on the anti-cancer activity of BSEs on other types of cancer. The proliferation of hepatocellular carcinoma cells, HepG2 and Hep3B, was inhibited by BSE with the following EC_{50s}: 21.21 ± 0.92 and 18.65 ± 0.71 µg/mL, respectively (Khan *et al.*, 2014), which are similar to the reported EC_{50s} in our project for A2780 (14 ± 2 µg/mL) and for A2780cis (11.5 ± 3 µg/mL) despite the differences in the cell lines, extraction solvents (methanol vs ethanol) and exposure time (72 h vs 24 h). They also stated that AKBA was 5 % w/w and KBA about 2 % w/w of the prepared extract, which suggests the involvement of other bioactive molecules in the cytotoxicity of the extract. They also observed an increase in caspase 3 activity and in the levels of tumour necrosis factor alpha (TNFα) and Interleukin 6 (IL-6) combined with a decrease in NFκB protein expression in the cells treated with BSE

indicating to the strong involvement of apoptosis induction in the reported anti-cancer activity of the extract. In addition, it was reported that BSE was effective against the induced colon cancer in a mouse model, where tumour formation was significantly reduced in animals supplemented with BSE in their daily diet compared to the control group (Chou *et al.*, 2017). Based on the present results and the discussed findings by the other researchers, it could be useful to assess BSEs interaction with chemotherapies or the interaction between single components of the extracts *in vitro* and *in vivo* using these ovarian cancer models.

The efficacy of AKBA on the tested cell lines (A2780, A2780cis, A2780ADR and OVCAR4) was similar in spite of the supposed molecular variability between them. It was as toxic towards the resistant cell lines A2780cis and A2780ADR as it was to their sensitive parent A2780 after 24 h with EC_{50s}: 35.64 ± 0.78, 30 ± 2 and 30.76 ± 1.71 μM, respectively. Moreover, it was also toxic to the HGSOC model, OVCAR4, with a slightly higher EC_{50} (40.3 ± 4 μM). The reported toxicity of AKBA on A2780 (EC_{50} ~22 μM) and A2780cis (EC_{50} ~ 18.5 μM) by Al-Salmani was slightly higher, which could be caused by the difference in the way the data was generated. The range of the doses used by Al-Salmani was between 5 and 50 μM (a total of 5 doses), whereas the range in the present project was between ~ 4 and 72 μM (a total of 8 doses). Comparable results were reported on other cell lines by other researchers. AKBA was effective against a Taxol resistant human ovarian cancer cell line A2780/Taxol with the EC_{50} ~ 51 μM (Jin *et al.*, 2019). In a study by Li *et al.* (2018), the growth of U251 and U87-MG, human glioblastoma cell lines, was inhibited by exposing cells to AKBA for 24, 48 and 72 h, where the EC_{50} values were reduced overtime. They were, respectively, ~ 28, 22 and 19 μM for U251 cells and ~ 32, 24 and 19 μM for U87-MG cells (Al-Salmani, 2017). This was not the case in the current project when cells exposed to AKBA for 48 h, where no changes to the EC_{50} was observed (data not shown). Considering the effectiveness of AKBA in reducing cancer cells viability based on current findings and the given examples of published articles, the impact of AKBA on the cell cycle, $\Delta\psi_m$, NFκB pathway, Nrf2/HO-1 pathway, ROS generation and P-gp expression was investigated attempting to understand the possible molecular mechanisms and targets that contribute to its cytotoxicity and its interaction with the selected chemotherapies.

It is clear that AKBA interferes with ovarian cancer cell proliferation by inducing cell cycle arrest at G0/G1 phase in A2780 cells that were exposed to AKBA for 24h. This was observed at the highest tested dose (62 μM) but not the other two doses (15 and 31 μM), which may mean that the other two doses require more time to exert their effect on the cell cycle in a

detectable level. Similarly, AKBA-induced cell cycle arrest at G1 was reported in colon cancer cells (Liu, Huang and Hooi, 2006) and prostate cancer cells (Yuan *et al.*, 2008), which was associated with increased expression of p21 and decreased expression of G1 phase cyclins (cyclin D1 and cyclin E) and CDKs 2 and 4. They state that the up-regulated expression of p21 was independent on the p21 gene potent transactivator, p53, considering the reported observation that AKBA downregulated p53 expression. Consistent with their findings, cell cycle was arrested at G1 and the expression of p53 was reduced by AKBA in the present project (see chapters 3 and 4). Further investigations are required to determine the mechanisms in which AKBA initiates cell cycle arrest at G1 by examining the molecular changes on both genetic and protein levels. Exploring this effect at various time points with a wider range of doses could lead to a better understanding of the impact of AKBA on the cycle. The effect of AKBA on the cell cycle coincided with the accumulation of DNA fragments at sub-G1, which will be discussed with cell death in the next paragraph.

As discussed earlier, the accumulation of fragmented DNA at sub-G1 in the cell cycle analysis is a widely used as marker of apoptosis since the internucleosomal DNA fragmentation is one of the hallmarks of apoptosis. However, it is stressed that DNA accumulation at sub-G1 must not be used solely as an apoptosis marker, should be interpreted with caution and other confirmative, more specific, tests should be considered (Kajstura *et al.*, 2007). The percentage of the events at sub-G1 was ~ 8 fold higher in the cells exposed to 62 µM AKBA for 24 h than in the untreated cells. This was not reported in the other tested two doses (15 and 31 µM) at this time point, which could be explained by either that the lower doses require more time or by the lack of reliability of using DNA hypodiploid (sub-G1) as an indicator of apoptosis. The former explanation is more apparent considering that changes in the $\Delta\psi_m$ could only be seen at around 50 µM and above. In a study by Hoernlein *et al.* (1999), sub-G1 peaks were observed after 4 h of exposing HL-60 cell to 50 µM AKBA, which was interpreted by the ability of AKBA to induce spontaneous apoptosis. In contrast, it was suggested that AKBA did not trigger apoptosis in a prostate cancer cell line, LNCaP, based on the unchanged percentage of sub-G1 compared to the untreated control even though cells were exposed to 30 µM (EC_{50} ~ 20 µM) AKBA for 48 h (Yuan *et al.*, 2008). The other possibility is that dead cells may not be present in the analysis and lost in the washes. This confirms the importance of using other more specific tests to explore the effects of tested agents on apoptosis. The impact of AKBA, cisplatin, doxorubicin and either combined with

AKBA on $\Delta\psi_m$ were explored to be used in the present project as an indicator of the induced apoptosis.

The use of $\Delta\psi_m$ as an indicator of mitochondrial health and as a marker of apoptosis is valid due to the direct and indirect involvement of mitochondria in several cellular processes, such as heat generation, redox and pH microenvironments regulation, proliferation and cell death, and for the inherited function of mitochondria in the intrinsic apoptotic pathway (Suzuki-Karasaki, Ochiai and Suzuki-Karasaki, 2014; Zorova *et al.*, 2018; Sivandzade, Bhalerao and Cucullo, 2019). Confirming the observations in sub-G1, AKBA led to mitochondrial depolarisation indicating to the involvement of impaired mitochondria in AKBA-induced cytotoxicity, likely through the intrinsic apoptotic pathway. This effect, similar to cell cycle, was observed at the high doses of AKBA at this time point (24 h) suggesting the importance of the time of exposure and the dose in targeting the mitochondria, which appears to be the two pivotal factors through the findings in this project. Further analysis of mitochondrial protein expression and activity could provide a better understanding of the impact of AKBA on mitochondria and their role in the AKBA-induced cancer cell cytotoxicity.

NFκB pathway has emerged as one of the targeted pathways in cancer therapy in view of the fact that it plays critical role in tumorigenesis and tumour progression in addition to its role in inflammation, which is one of hallmarks of cancer, and other physiological and pathological processes (Hoesel and Schmid, 2013; Park and Hong, 2016; Xia *et al.*, 2018). The anti-inflammatory effects of boswellic acids are attributed, in part, to their ability to inhibit NFκB activity through suppressing IKK (Patlolla and Rao, 2011). The ability of AKBA to inhibit NFκB signalling was described as a potential avenue of the mechanism of actions of AKBA against cancer cells (Qurishi *et al.*, 2010). It is evident, based on the NFκB pathway proteome profiler, that AKBA downregulates NFκB pathway by targeting a variety of proteins including NFκB activators, transcription factors and receptors (see chapter 4). This preliminary effect needs to be confirmed in further experiments with wider range of doses and multiple time points. Moreover, it could be beneficial to explore the impact of the agents on the activity of the pathway. Considering the involvement of NFκB signalling in tumorigenesis and tumour survival, AKBA could be further explored as an agent to either prevent or treat cancer since NFκB has been consistently established as one of targets of AKBA. It is, though, crucial to avoid long time global knockdown of NFκB considering its importance in inflammation and infection fighting.

6.2 AKBA interaction varies depending on the combined chemotherapy

6.2.1 AKBA antagonises with cisplatin and adds to/antagonises with paclitaxel

In this section of the chapter, the interaction of AKBA with paclitaxel or cisplatin will discussed. Although the EC_{50s} of paclitaxel were improved 10 times on A2780 and 2 times on A2780cis, the CI values indicated for additive effect on A2780 and moderate antagonism on A2780cis. This observed results could be improved by increasing the exposure time to be 72 h rather than 24 h or by examining the effect of combination of AKBA and paclitaxel on a paclitaxel resistant cell line, A2780/Taxol (Jin *et al.*, 2019). This approach could be supported by the decreased expression of P-gp by AKBA when combined with doxorubicin, which was mentioned by Jin *et al.* (2019) as one of the reasons behind the ability of AKBA to sensitise A2780/Taxol to paclitaxel. Moreover, ursolic acid, a pentacyclic triterpene, sensitised paclitaxel resistant breast cancer cells to paclitaxel by targeting miR-149-5p/MyD88 (Xiang *et al.*, 2019). Even though the expression of MyD88 was low in A2780, AKBA reduced its expression to about the half of the untreated control (see Chapter 4). Cisplatin was chosen for further analysis since the antagonism with AKBA appeared higher than in the case of antagonism between paclitaxel and AKBA and for the problems associated with paclitaxel solubility and delivery such as its high affinity towards the membrane bound P-gp efflux pump, which contributes to the poor clinical outcomes (Gursoy *et al.*, 2003; Surapaneni, Das and Das, 2012).

The interaction between AKBA and cisplatin is antagonistic based on MTT data and CI values of the tested cell lines, A2780, A2780cis and OVCAR4. The antagonism between AKBA and cisplatin was not seen in the cell cycle analysis in A2780 at the high doses of cisplatin 15 and 23 µM, where cisplatin arrested cells at S phase in the presence or the absence of AKBA. However, the effect of cisplatin on the cell cycle at 12 µM was reduced in the presence of AKBA. These observations suggest that the AKBA interfere with cisplatin impact on the cell cycle in a dose and time dependent manner, assuming that similar effect with the highest doses will be recognised if the exposure was less than 24 h. In addition, the percentages of fragmented DNA at sub-G1 was less by about two-fold in the cells exposed to cisplatin combined with AKBA compared to the cells exposed to cisplatin alone.

The impact of cisplatin alone and combined with AKBA on Nrf2 and HO-1 proteins expression was examined. Both AKBA alone and cisplatin alone have not affected the expression of the proteins at 24 h, whereas cisplatin decreased their expression at 1 h.

Knocking down Nrf2 or inhibiting its activity could be used in the future to test its involvement in the antagonistic interaction between AKBA and cisplatin, which may then result in improving their cytotoxicity. Finally, Changing the treatment approach from co-exposure to pre-exposure was suggested as a possible solution, in theory (Sirota, Gibson and Kohen, 2017), to overcome the reported antagonism and more importantly may enhance cisplatin cytotoxicity leading to synergistic interaction between AKBA and cisplatin.

6.2.2 Sensitising ovarian cancer cells to doxorubicin using AKBA

In another attempt to establish the possible interaction between AKBA and chemotherapeutic agents, AKBA remarkably enhanced the cytotoxicity of doxorubicin against all tested cell lines, A2780, A2780cis, A2780ADR and OVCAR4. This, interestingly, includes sensitizing the resistant cell line A2780ADR to doxorubicin. This synergistic interaction between AKBA and doxorubicin could be attributed to the fact that both agents target multiple mechanisms on their own or combined (Figure 6.1). The first explored mutual targeted mechanism was the cell cycle, where arresting cell cycle at G2/M phase by doxorubicin was not affected by the presence or the absence of AKBA.

On the other hand, AKBA altered the effect of doxorubicin on $\Delta\psi_m$ from no effect/apparent hyperpolarisation to depolarisation, which may mean that both induce apoptosis through disrupting the mitochondria, which activates the intrinsic apoptotic pathway (Ly, Grubb and Lawen, 2003; Gaafary *et al.*, 2014). The other possible target pathway is NFκB pathway, where the expression of a number of proteins were affected by both agents. The crosstalk between mitochondrial ROS and depolarisation was found to selectively induce the extrinsic apoptotic pathway in cancer cells through upregulating TRAIL protein expression, which binds to TRAIL R2/DR5 (Suzuki-Karasaki, Ochiai and Suzuki-Karasaki, 2014). Further analysis to determine the exact role and key players of NFκB components in the synergistic interaction between AKBA and doxorubicin is required. NFκB knockout using CRISPR/CAS9-mediated gene editing (Wang *et al.*, 2018) could reveal the function of other members of the pathway in the interaction. The other recommended approach is to increase the range of the doses of both agents and test various times of exposure. Furthermore, examining NFκB pathway in A2780ADR may explain the sensitisation to doxorubicin that resulted from combining AKBA with doxorubicin since NFκB constitutive activation was linked to chemoresistance in prostate cancer (Qurishi *et al.*, 2010).

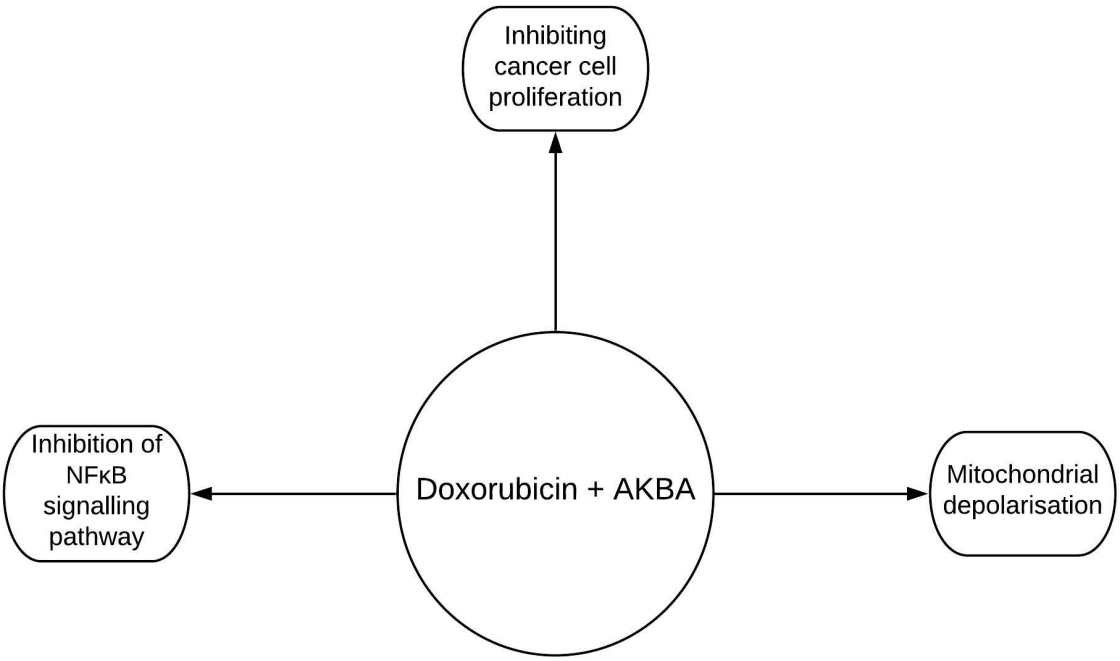


Figure 6.1: A summary of the synergistic interaction between AKBA and doxorubicin.

Finally, P-gp was one of the suggested reasons for the synergistic interaction. The effect of doxorubicin alone or combined with AKBA was not statistically significant at the tested doses and time points. It may mean that P-gp is not involved in the interaction between AKBA and doxorubicin or the doses and time points are not enough to reveal its role. P-gp activity determination or expression knock down (Norouzi-Barough *et al.*, 2018) is a necessary approach test the involvement of P-gp in the synergistic interaction between AKBA and doxorubicin.

These promising findings are encouraging for further investigations that could lead to use of AKBA or BSE as an adjuvant in cancer chemotherapy, which could also help to reduce side effects of chemotherapies. Exploring the involvement of other mutual targets of AKBA and doxorubicin, that were not examined in this project, could lead to a better understanding of the synergistic interaction between them. For example, inhibition of topoisomerases I and II was described as one of the mechanisms behind the cytotoxicity of both AKBA and doxorubicin against cancer cells (Syrovets *et al.*, 2000; Mizutani *et al.*, 2005; Ni *et al.*, 2012; Zhu *et al.*, 2016).

6.3 BSE and AKBA *in vivo* and in the clinical trials

The anti-inflammatory effect of BSE and BAs, and the role of inflammation in tumorigenesis and cancer progression has encouraged researchers to investigate their potential efficacy against cancer as discussed throughout this project. A number of pre-clinical, on *in vivo* models, and clinical studies will be discussed here.

A number of *in vivo* studies have confirmed the efficacy of various extracts, essential oils or single bioactive molecules, AKBA in particular, from frankincense against different types of cancer. Anti-cancer activities were reported on pancreatic cancer (Syrovets, Gschwend, *et al.*, 2005; Park *et al.*, 2011; Ni *et al.*, 2012), glioma (Winking *et al.*, 2000; Conti *et al.*, 2018), prostate cancer (Liu *et al.*, 2019) and colorectal cancer (Takahashi *et al.*, 2012; Yadav *et al.*, 2012; Chou *et al.*, 2017) in animal models confirming previous findings on *in vitro* models. This was attributed to modulation of various molecular targets, such as inhibition of NFκB pathway, and to the induction of apoptosis in cancer cells. Despite this effort in the mentioned examples of *in vivo* studies, the promising anti-cancer activity of BSE deserves further investigation in a hope of translating this into clinical use to help in the treatment of cancer.

With regards to clinical trials, a handful of clinical trials were carried out on the effect of BSE on chronic diseases, mainly inflammatory conditions. In a review by Roy *et al.*, (2019), BSE in clinical trials was effective on the following diseases: osteoarthritis, knee arthritis, gonarthrosis, brain tumour related oedema, photoaged skin, Crohn's disease, diabetes erythematous eczema and asthma. Importantly, this was combined with indications that BSE is safe and no serious side effects were reported in a number of studies (Liu *et al.*, 2002; Moussaieff and Mechoulam, 2009; Azadmehr *et al.*, 2014; Roy *et al.*, 2019). It is hard to find clinical trials of BSE on cancer. The nearest form of clinical use of BSE on cancer is a case report of a successfully treated basal-cell carcinoma of the skin by a topical application of frankincense essential oil that was extracted by hydrodistillation (Fung *et al.*, 2013). There seems to be a lack of clinical trials, especially on cancer, combined with insufficient *in vivo* studies even though frankincense has been shown to possess promising anti-tumour properties on a broad range of cancer models. Further investigations of the possible applications of BSE on cancer in pre-clinical and clinical settings are needed taking into account current findings and published evidence on the anti-cancer potentiality of BSE and boswellic acids.

6.4 Conclusion

This project demonstrates the potential anti-tumour activity of BSE and AKBA, against ovarian cancer in particular. This confirms similar observations reported on other cancers. The interaction between AKBA and chemotherapies seems to be dependent on a number of factors including cell line, time of exposure and doses of AKBA or the doses of a standard chemotherapeutic drug. The key observation is the ability of AKBA to remarkably improve the cytotoxicity of doxorubicin towards ovarian cancer, including sensitising the resistant cells to doxorubicin. The combination of AKBA with doxorubicin could lead to a novel treatment strategies for ovarian cancer that could help in reducing the side effects of doxorubicin and improving its efficacy.

6.5 Limitations and future work

The first limitation to note is the range of doses or time points used in the experiments. A wider range could provide a better understanding. For example, testing the effect of AKBA on the cell cycle at different time points, i.e. 1, 6, 12, 48, 72 h, could help in revealing the time where a specific dose of AKBA triggers changes in the cell cycle, which in turn helps in future mechanistic exploration when examining involved molecular changes such as the expression of proteins involved in the cell cycle regulation. The other limiting factor that is linked to this point is using a single dose of AKBA, that reduces cell viability to 60/70 %, especially in the agent interaction experiments and mechanistic experiments. The same applies for other experiments performed in this project.

With regards to protein expression experiments, it would have been useful to collect samples in a different way to gather proteins in the extracellular environment (the medium) rather than cellular proteins alone.

Using adherent cell culture (two-dimensional, 2D) in this project is a reason that may restrict some conclusions drawn. One of the suggestions to overcome this limitation is using three-dimensional (3D) ovarian cell culture model (spheroids), which is considered as a relatively better *in vitro* model (Yang and Zhao, 2011). It is reported that 3D cell culture model of ovarian cancer showed alterations in the protein expression and more resistance to chemotherapies, which suggests that 3D cell culture is superior model in in terms of mimicking the *in vivo* conditions compared to conventional 2D cell culture (Yang and Zhao, 2011; Lee *et al.*, 2013).

Findings of the present project on the potent toxicity of BSE and AKBA on ovarian cancer combined with the dual interaction of AKBA with chemotherapies, in particular the synergism with doxorubicin, confirm previously published data on other cancer types. This warrant further pre-clinical investigations, which form the basis for future clinical trials. Here is a number of suggestions for future work:

- Further mechanistic analysis is required to confirm and understand the molecular events behind the reported interaction between AKBA and doxorubicin.
- The toxicity of doxorubicin was enhanced by combining single dose of AKBA, it is interesting to explore whether the toxicity of AKBA will be enhanced by doxorubicin.
- After establishing the potential anti-tumour activity of the ethanol extract in this project, various extracts of frankincense could be further investigated in combination with selected chemotherapies.
- Similar approaches of treatments using AKBA or BSE alone or combined with chemotherapies could be applied on 3D cell culture or on *in vivo* models.
- Pre-clinical studies to establish ADME (absorption, distribution, metabolism, and excretion) and pharmacokinetics (PK) are vital prior phase I clinical trials in humans.
- Since stem cells play critical role in chemoresistance, it could be beneficial to investigate the effect of BSE or AKBA alone or combined with chemotherapies on CD44+/MyD88 ovarian cancer stem cells (Alvero *et al.*, 2011). This could open new avenues in treating resistant ovarian cancer or on preventing the recurrence of the disease.

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Appendix

Thin layer chromatography (TLC)

To conduct a preliminary examination of the presence of AKBA in the frankincense extracts, TLC was used. Fluorescent TLC plates were used and the mobile phase consisted of 7 parts of hexane, 3 parts of ethyl acetate and one drop of formic acid. A spot of the oily component and the clear phase of the 70 % ethanol extract, and a drop of pure AKBA (9.75 mM) were placed on the TLC plate, about 1 cm from the bottom of the plate. The plate was put in the chromatography tank and the plate run until the solvent was approximately 0.5 cm from the top of the plate, the solvent front was marked and the solvent allowed to dry. The plate was placed in a mini UV viewing cabinet (UVP, model: C-10, Cambridge, UK) to mark the AKBA position and compare to the component from the two examined extracts. To be able to visualise AKBA and other compounds by naked eye, the plate was sprayed using anisaldehyde spray which is composed of 0.5 mL anisaldehyde, 85 mL methanol, 10 mL acetic acid and 5 mL sulphuric acid.

TLC chromatogram

The presence of AKBA in the purified extracts was tested using TLC. The possible presence of AKBA wasn noted in addition to other un-identified compounds separated in both extracts. Retention factors (R_f) of the control AKBA (middle of spot) was 0.37. Similar R_f values were found in the oily and clear extracts 0.4 and 0.42, respectively (Figure 6). Further identification and quantification of the compounds using gas chromatography–mass spectrometry (GC-MS) and/or high-performance liquid chromatography (HPLC) are required (Ni *et al.*, 2012).

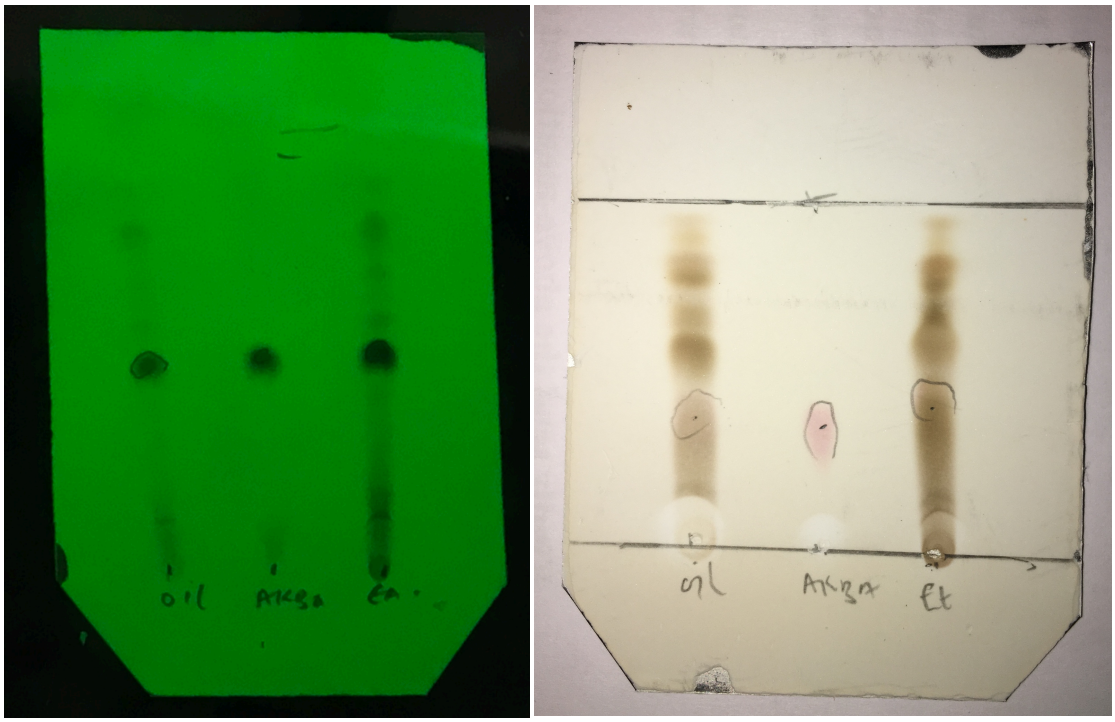


Figure 6: UV detection (left) and a chromatogram detection (right) of AKBA and other compounds in the extracts.

Δψm assay (JC-10) protocol:

(https://www.abcam.com/ps/products/112/ab112134/documents/ab112134%20JC-10%20Mitochondrial%20Membrane%20Potential%20Assay%20Kit%20Microplate%20v7b%20(website).pdf)

A. Preparation of Cells

1. For adherent cells: Plate cells overnight in growth medium at 20,000 to 80,000 cells/well/90 µL for a 96- well plate or 5,000 to 20,000 cells/well/20 µL for a 384- well plate.

2. For non-adherent cells: Centrifuge the cells from the culture medium and then suspend the cell pellet in culture medium at 100,000-200,000 cells/well/90 µL for a 96-well poly-D lysine plate or 25,000 - 50,000cells/well/20 µL for a 384-well poly-D lysine plate. Centrifuge the plate at 800 rpm for 2 minutes with brake off prior to the experiments

Note: Each cell line should be evaluated on the individual basis to determine the optimal cell density for apoptosis induction.

B. Preparation of JC-10 dye-loading solution

1. Thaw all the kit components at room temperature before use.

2. Add 50 µL of 100X JC-10 (Component A) into 5 mL Assay Buffer A (Component B), and mix well.

Note: Aliquot and store the unused Component A at -20°C. Avoid repeated freeze/thaw cycles.

C. Run JC-10 Assay

1. Treat cells by adding 10 µL of 10X test compounds (96- well plate) or 5 µL of 5X test compounds (384-plate) into the desired buffer (such as PBS or HHBS).

Note: It is not necessary to wash cells before adding compound. However, if tested compounds are serum sensitive, growth medium and serum factors can be aspirated away before adding compounds. Add the same volume of HHBS into the wells (such as 90 µL for a 96-well plate or 20 µL for a 384-well plate) after aspiration. Alternatively, cells can be grown in serum- free media.

2. Incubate the cell plate at room temperature or in a 37 °C, 5% CO2 incubator for at least 15 minutes or a desired period (4-6 hours for Jurkat cells treated with camptothecin) to induce apoptosis.

3. Add 50 µL/well (96-well plate) or 12.5 µL/well (384-well plate) of JC-10 dye-loading solution (from step B.2) into the cell plate (from Step C.2).

4. Incubate the dye-loading plate at room temperature or in a 37°C, 5% CO2 incubator for 30 minutes to 1 hour, protected from light.

Note: The appropriate incubation time depends on the individual cell type and cell concentration used. Optimize the incubation time for each experiment.

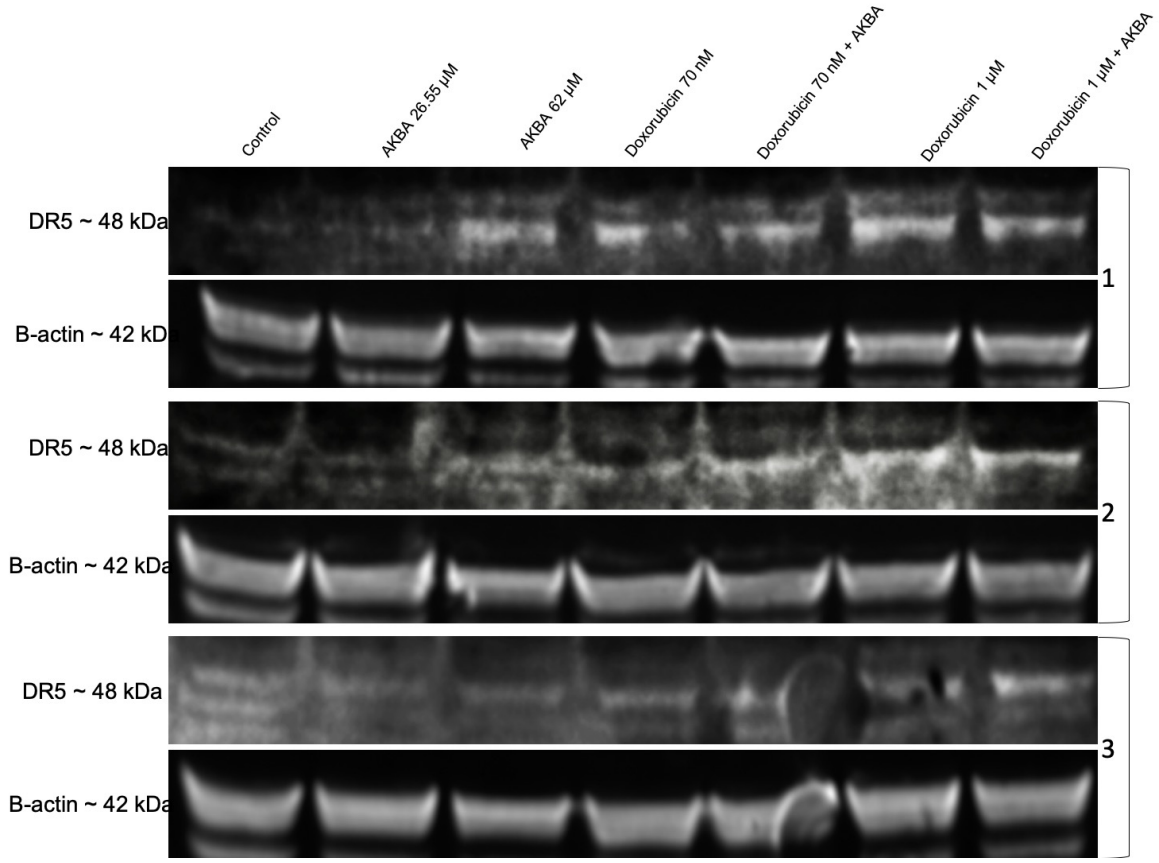
5. Add 50 µL/well (96-well plate) or 12.5 µL/well (384-well plate) of Assay Buffer B (Component C) into the dye- loading plate (from Step C.4) before reading the fluorescence intensity.

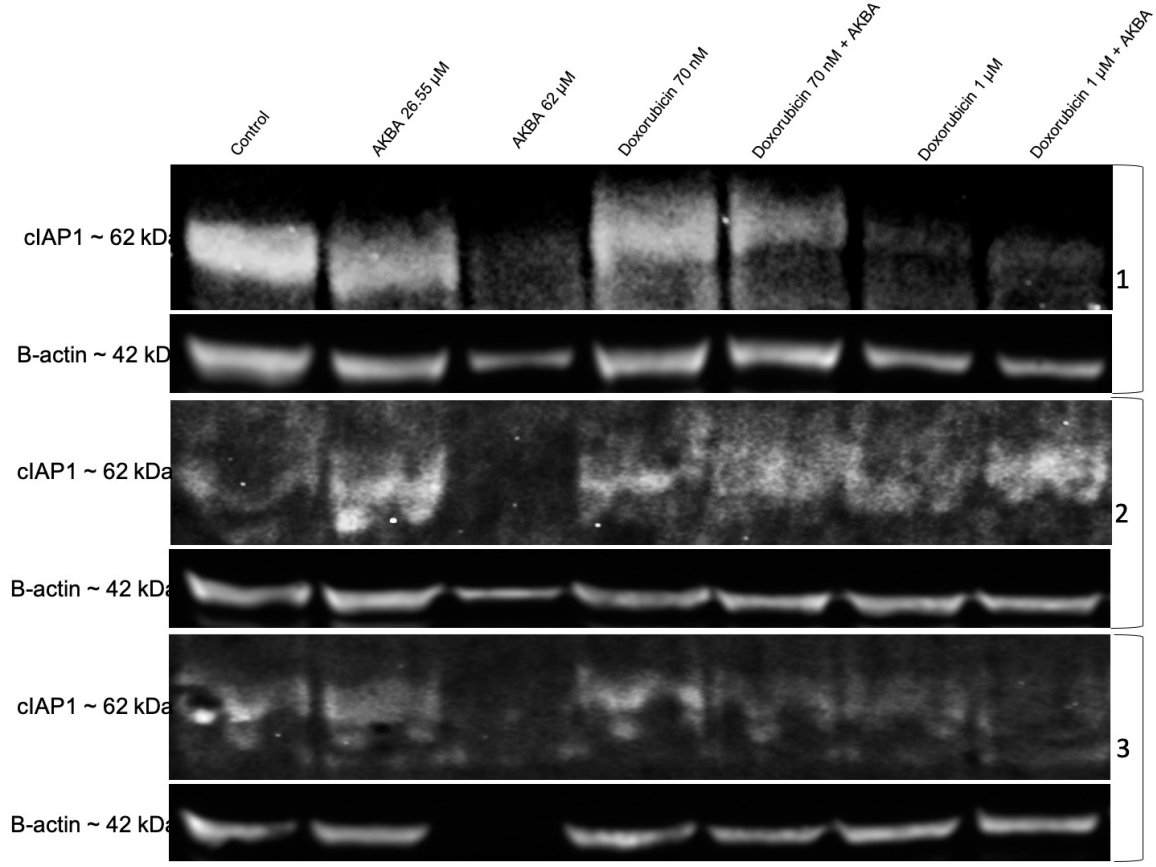
Note 1: DO NOT wash the cells after loading.

Note 2: For non-adherent cells, it is recommended to centrifuge cell plates at 800 rpm for 2 minutes with brake off after adding Assay Buffer B (Component C).

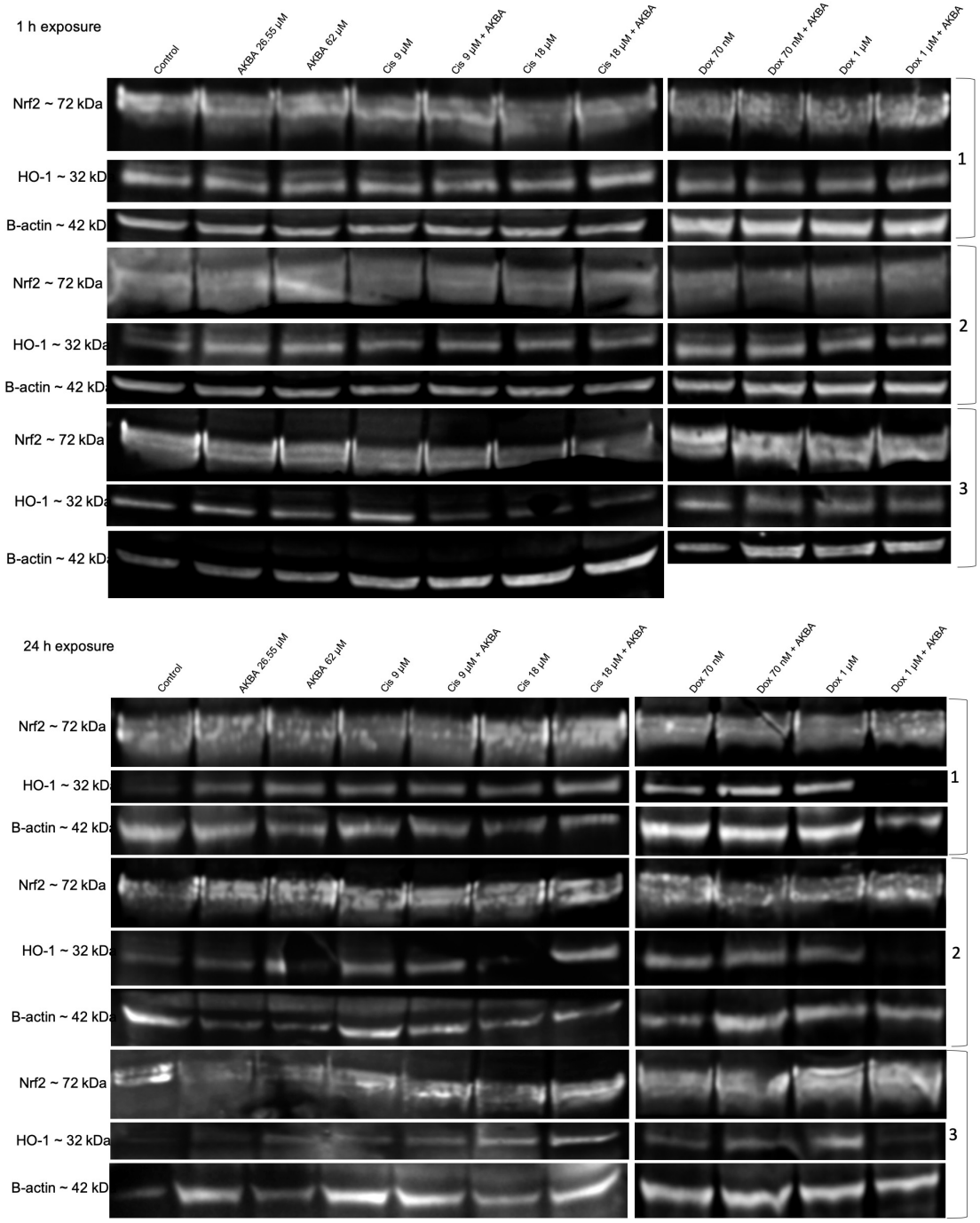
6. Monitor the fluorescence intensities at Ex/Em = 490/525 nm (cut off at 515 nm) and 540/590 nm (cut off at 570 nm) for ratio analysis.

DR5 and cIAP1 replicates:

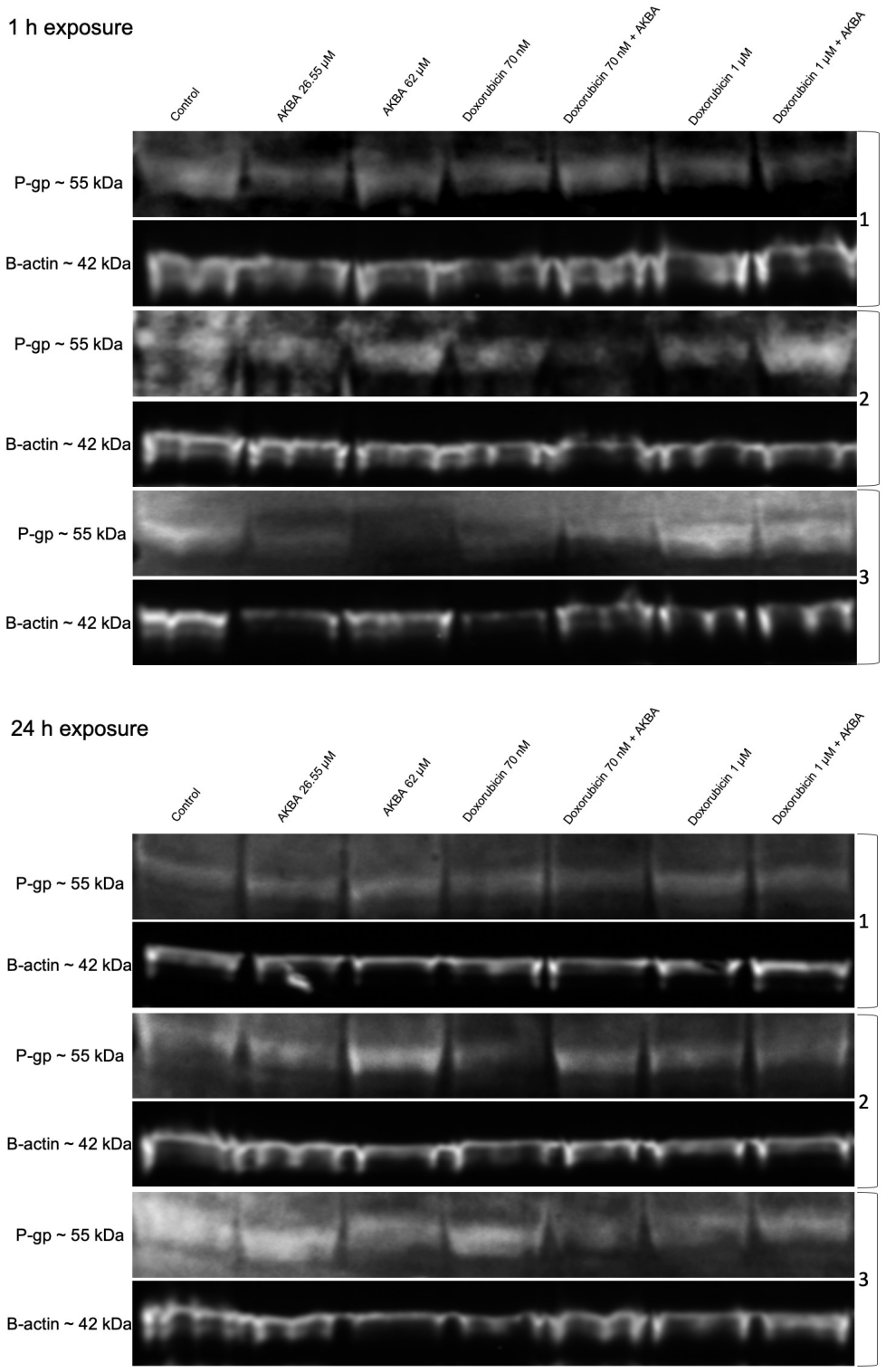




Nrf2 and HO-1 replicates n=3:



P-gP replicates n=3:



Proteome Profiler™ Array Human NFκB Pathway Array Kit

Protein list:

ASC	IL-18 R alpha	RelA/p65 (pS529)
BCL-10	IRAK1	c-Rel
CARD6	IRF5	SHARPIN
CD40/TNFRSF5	IRF8	SOCS-6
dAP1/BIRC2	JNK1/2	STAT1p91
dAP2/BIRC3	JNK2	STAT1 (pY701)
FADD/MORT1	LTBR/TNFRSF3	STAT2
Fas/TNFRSF6/CD95	Metadherin/AEG-1	STAT2 (pY689)
IκBα	MYD88	STING/TMEM173
IκBε	NFκB1	TLR2
IKK1/IKKα/CHUK	NFκB2	TNF RI/TNFRSF1A
IKK2/IKKβ	NGF R/TNFRSF16	TNF RII/TNFRSF1B
IKKυ/NEMO	P53	TRAF2
IL-1 RI	P53 (pS46)	TRAIL R1/DR4
IL-17 RA	RelA/p65	TRAIL R2/DR5

ARRAY PROCEDURE

Bring all reagents to room temperature before use. Keep samples on ice. To avoid contamination, wear gloves while performing the procedures.

1. Prepare all reagents and samples as directed in the previous sections.
2. Pipette 2.0 mL of Array Buffer 3/6 into each well of the 4-Well Multi-dish to be used. Array Buffer 3/6 serves as a block buffer.
3. Using flat-tip tweezers, remove each membrane to be used from between the protective sheets and place in a well of the 4-Well Multi-dish. The array number should be facing upward.

   Note: *Upon contact with Array Buffer 3/6, the blue dye from the spots will disappear, but the capture antibodies are retained in their specific locations.*

4. Incubate for one hour on a rocking platform shaker. Orient the tray so that each array rocks end to end in its well.
5. While the membranes are blocking, prepare samples by adding the desired quantity of lysate to Array Buffer 1 for a total volume of 1.5 mL. Maximum lysate volume is 250 µL.
6. Aspirate Array Buffer 3/6 from the wells of the 4-Well Multi-dish. Add prepared samples and place the lid on the 4-Well Multi-dish.
7. Incubate overnight at 2-8 °C on a rocking platform.

   Note: *A shorter incubation time may be used if optimal sensitivity is not required.*

8. Carefully remove each membrane and place into individual plastic containers with 20 mL of 1X Wash Buffer. Rinse the 4-Well Multi-dish with deionized or distilled water and dry thoroughly.
9. Wash each membrane with 1X Wash Buffer for 10 minutes on a rocking platform shaker. Repeat two times for a total of three washes.
10. For each array, dilute 15 µL of reconstituted Detection Antibody Cocktail to 1.5 mL with Array Buffer 3/6. Pipette 1.5 mL per well of diluted Detection Antibody Cocktail into the 4-Well Multi-dish.
11. Carefully remove each membrane from its wash container. Allow excess buffer to drain from the membrane. Return the membrane to the 4-Well Multi-dish containing the diluted Detection Antibody Cocktail. Cover the wells with the lid.
12. Incubate for 1 hour at room temperature on a rocking platform shaker.

13. Wash each array as described in steps 8 and 9.

14. Dilute the Streptavidin-HRP in Array Buffer 3/6 using the dilution factor on the vial label. Pipette 2.0 mL into each well of the 4-Well Multi-dish.

15. Carefully remove each membrane from the wash container. Allow excess Wash Buffer to drain from the membrane. Return the array to the 4-Well Multi-dish containing the diluted Streptavidin-HRP, and cover with the lid. Incubate for 30 minutes on a rocking platform shaker.

16. Wash each array as described in steps 8 and 9.

Note: *Complete the remaining steps without interruption.*

17. Carefully remove each membrane from its wash container. Allow excess Wash Buffer to drain from the membrane by blotting the lower edge onto paper towels. Place each membrane on the bottom sheet of the plastic sheet protector with the identification number facing up.
18. Pipette 1 mL of the prepared Chemi Reagent Mix evenly onto each membrane.

    Note: *Using less than 1 mL of Chemi Reagent Mix per membrane may result in incomplete membrane coverage.*

19. Carefully cover with the top sheet of the plastic sheet protector. Gently smooth out any air bubbles and ensure Chemi Reagent Mix is spread evenly to all corners of each membrane. Incubate for 1 minute.
20. Position paper towels on the top and sides of the plastic sheet protector containing the membranes and carefully squeeze out excess Chemi Reagent Mix.
21. Remove the top plastic sheet protector and carefully lay an absorbent lab wipe on top of the membranes to blot off any remaining Chemi Reagent Mix.
22. Leaving membranes on the bottom plastic sheet protector, cover the membranes with plastic wrap taking care to gently smooth out any air bubbles. Wrap the excess plastic wrap around the back of the sheet protector so that the membranes and sheet protector are completely wrapped.
23. Place the membranes with the identification numbers facing up in an autoradiography film cassette.

    Note: *Use an autoradiography cassette that is not used with radioactive isotope detection.*

24. Expose membranes to X-ray film for 1-10 minutes. Multiple exposure times are recommended.

(https://resources.rndsystems.com/pdfs/datasheets/ary029.pdf)